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Association between proliferation status of infected and non-infected mononuclear cells with tissue lesions in acute bovine theileriosis

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Abstract

Tropical or Mediterranean theileriosis in dairy cattle is widely distributed in many tropical regions of the world. The purpose of this study was to evaluate the proliferation status of mononuclear cells infected with *Theileria annulata* schizonts in different tissues and its relationship with the pathogenesis of the parasite in cattle by histopathology, immunohistochemistry and polymerase chain reaction (PCR). Blood and tissue samples of eight Holstein cattle that had been lost due to theileriosis and eight healthy slaughtered cattle of the same breed were collected as a control group after necropsy. The piroplasms in the blood smears and the schizonts in the cytoplasm of the lymphocytes and macrophages of the lymph nodes were microscopically detected. Histopathologically, the proliferation of macrophages, lymphocytes, and plasma cells in lymph nodes and the heart, congestion, and bleeding in the red pulp of the spleen, portal tracts of the liver, interstitial tissue of the kidneys, multifocal necrosis and ulceration in the abomasum together with hyperemia and hemorrhages and lymphoblastic infiltration in the submucosa and lamina propria adjacent to these lesions and emphysema with ecchymotic hemorrhage in the lungs were evident. Immunohistochemistry identified the proliferated cells as mostly Cluster of Differentiation 3- Positive T lymphocytes and macrophage marker antibody 387- positive macrophages. Positive results of PCR for the *Tams1* 30.00 kDa gene were observed in lymph nodes, liver, lung and abomasum. It was concluded that the pathological changes were the result of schizont-infected macrophage proliferation leading to severe uncontrolled proliferation of uninfected T lymphocytes.

Introduction

The *Theileria* parasites belong to the phylum *Apicomplexa*. The genus *Theileria* includes a number of protozoa species transmitted by ticks found in ruminants and other mammals. Tropical or Mediterranean theileriosis is an economically important disease that is prevalent in North Africa, southern Europe, India, the Middle East, and Asia; an estimated 200 million cattle are at risk.[1] This disease is caused by the protozoan parasite *Theileria annulata*, affecting the domestic cattle *Bos Taurus* and *Bos indicus* and the Asian buffalo (*Bubalus bubalis*). It is transmitted by several species of *Hyalomma* spp. ticks.[2] The disease is widespread and well known in several parts of the Middle East including Iran, and it affects animals' husbandry and their productions in the country.[3-4] The severity of the disease varies from limited clinical reactions in sustained endemic areas to more complicated disease in unstable endemic areas leading to high mortality calves. Primarily, the pathogenesis is due to the proliferation of infected leukocytes and anemia caused by the destruction of red blood cells.[5]

Infection of cattle is the result of inoculation with *T. annulata* sporozoites during infected tick feeding. Macrophages and sometimes B lymphocytes can be infected by sporozoites which rapidly invade host lymphocytes and mature into multinucleate macroschizonts. The infected leukocytes disseminate to various organs and tissues, provoking the division of uninfected cells and continuing to propagate as parasite-infected cells.[6]

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Following cattle infection, the disease can be observed in three forms: acute, sub-acute or chronic. The severity of the disease depends on the breed, age, immune status of the host and the number of sporozoites inoculated by the tick.[6] Cattle are resistant to re-infection after recovery.[6] The disease is typically sub-acute in endemic areas and in indigenous breeds.[6] Imported European breeds, however, are more susceptible compared with crossbred and indigenous animals and the disease appears acutely with high mortality.[6]

The pathological progression of the disease in an acute and often fatal infection occurs in three stages: incubation, acute and sub-acute. The most prominent symptoms are superficial lymph node enlargement, anorexia, pale mucous membranes, corneal opacity, emaciation, weakness, infertility, increased heart rate, and shortness of breath.[7]One of the characteristics of theileriosis is that animals recovering from an acute infection can carry the infectious agent for a long time, thus becoming a potential source of infection for healthy and susceptible animals.[8]

Theileriosis is a protozoan infection with economic importance among domestic cattle in Iran, with an overall infection rate of 19.00%. The disease is far-reaching and renowned in most parts of Iran affecting animals' husbandry and production. The highest rate of the disease took place from June to July and the lowest from March to April, in northwestern and southeastern Iran.[9,10] Moreover, the high prevalence of ovine theileriosis in different areas of Iran is recognized through several factors including vector seasonal rate, climatic and ecological changes, host vulnerability, ticks' resistance to insecticides, a high level of tick-infestation in sheep compared to cattle, and inadequate preventive policies.[9-11] Further investigation and monitoring will be needed to extend the supervision and control policies, such as vaccination, the development of traditional diagnostic tools and the assessment of pesticide resistance in ticks to reduce the mortality and morbidity of theileriosis among livestock and ultimately decrease the risk of outbreaks and economic loss as well as public health hazards in Iran.

Although some components of the immune response to *T. annulata* are well understood, the pathogenesis of the disease, including the potential role of ectopic immune responses, has not been largely identified. Macrophages are among the defense cells that can play a role in stimulating and weakening the immune system, thus changing the effect and characteristics of immune responses in various tissues.[8] This study can reveal whether the pathogenesis and clinical signs of bovine theileriosis are related to the proliferation of *T. annulata* schizont or the proliferation and invasion of mononuclear cells infected with this protozoan observed in different tissues of infected cattle. In this regard, using immuno-histochemistry, histopathology and molecular tools is necessary and significantly helpful in understanding the immunopathogenesis of tropical theileriosis and ultimately controlling and treating of the disease.

In most pathological studies on bovine theileriosis in Iran and other parts of the world, the presence of schizonts in stained tissue sections of lymphatic and non-lymphatic organs has been reported.[12-14] The aim of this study was to reveal the presence of small schizonts or merozoites in the affected areas, as well as to investigate the diffusion status of mononuclear cells among different tissues and its relationship with the pathogenesis of tropical theileriosis in cattle through histopathology, immunohistochemistry and polymerase chain reaction (PCR) methods.

Materials and Methods

Sampling. From 2019 to 2021, eight Holshtein cattle exhibiting signs of malignant theileriosis resulting in death or euthanasia were identified from the North-Eastern (5 cases) and North-Western (three cases) regions of Iran. All cattle were examined by a veterinarian prior to necropsy. The clinical signs included fever, enlarged lymph nodes, ocular and vaginal mucous membrane hemorrhages, jaundice, anorexia and diarrhea. The final confirmation of theileriosis was carried out by thin blood and lymph node smear staining technique. Samples were collected from different tissues including lymph nodes, liver, spleen, lung, kidney, heart, and abomasum of the affected animals and the control samples were prepared and stored in two containers: one containing 10.00% formalin buffer for histopathological and immunohistochemical examinations and another containing absolute ethanol for PCR analysis. Similar samples were also collected from eight healthy Holshtein cattle as a control group in the slaughterhouse. All animal experiments of the project (No. 3/49089) were performed in strict accordance with the guidelines approved by the Animal Ethics Committee of Ferdowsi University of Mashhad, Iran.

Blood and tissue smears. Methanol-fixed thin blood smears were prepared and stained using Giemsa, and studied under a light microscope (Olympus, Tokyo, Japan). Lymph node fine needle aspirates were obtained and stained with Giemsa and assessed for the presence of schizonts (Koch bodies) by examining random fields at 100× magnification.[15]

Histopathogical examination. For histopathology, formalin-fixed sections of lung, lymph node, heart, liver, spleen, kidney, and abomasum were processed and stained using Hematoxylin and Eosin (H&E), and microscopically examined using a light microscope.[16] Furthermore, a modified Giemsa stain (a member of the Romanowski group of stains) was used to visualize the schizonts in different tissue samples.

The DNA extraction and PCR. Prior to PCR, tissue samples reserved in ethanol were prepared for DNA

extraction. Using the Sinaclone Company extraction kit (Tehran, Iran), the DNAs were extracted. The oligonucleotide primers used to amplify *Tams1* 30.00 kDa gene sequences of *T. annulata* (721 bp) were as follows: (Forward N516) 5'GTAACCTTTAAAAACGT3' and (Reverse N517) 5'GTTACGAACATGGGTTT3'. *Theileria annulata* specific primers (N516/N517) derived from the gene encoding the 30.00 kDa major surface antigen of *T. annulata* merozoite (*Tams-1*) was used in the amplification reaction described by d'Oliveira *et al*.[17] The PCR was performed in a final reaction volume of 25.00 µL containing 12.50 µL Master mix (Biotechnology, Korea), 4.00 µL extracted DNA sample, 1.50 µL of each primer including forward and reverse (10.00 pmol μL^{-1}) and, 5.00 µL nuclease-free water. The PCR reaction performed in an automatic DNA thermal cycler (Eppendorf, Hamburg, Germany) involved an initial denaturation step at 94.00 ˚C for 3 min; 30 thermal cycles, each of which consisted of a denaturation step of 1 min at 94.00 ˚C, an annealing step of 1 min at 55.00 ˚C, and an extension step of 1 min at 72.00 ˚C; a final extension step of 10 min at 72.00 ˚C and a holding step at 4.00 ˚C until the samples were taken out from the thermal cycler. The electrophoresis (Fanavaran SahanAzar, Iran) of amplified PCR products was conducted on 1.00% agarose gel and then visualized and photographed. Genomic DNA of the positive control was extracted from a blood sample of an infected dairy cattle with *T. annulata* and the nuclease free water was used as a negative control for each PCR amplification.

Immunohistochemistry (IHC). Immunophenotyping of the leukocytes was performed using the immunohistochemical detection kit of ab64261® (Abcam, Tokyo, Japan). This is a labeled streptavidin-biotin immunoenzymatic antigen detection system (Abcam). This technique requires the successive incubation of the sample with an unconjugated primary antibody specific to the target antigen, the reaction of the primary antibody with a biotinylated secondary antibody, an enzyme-labeled streptavidin, and a substrate/chromogen. The basic conjugates were: (i) monoclonal conjugate (Rabbit anti-human Cluster of Differentiation (CD)-3) for T. cell detection, (ii) monoclonal conjugate (rabbit anti-human CD20) for B. cell detection, and (iii) conjugate for macrophage detection (anti-human macrophage marker antibody (MAC)-387).[18] The IHC protocol included: deparaffinization and rehydration of tissue sections fixed with formalin and paraffin-embedded, adding hydrogen peroxide to cover the sections and incubating for 10 min, washing in buffer, performing suitable pretreatment, washing in buffer, protein blocking and incubating for 10 min at room temperature to block nonspecific background staining, wash in buffer, applying the primary antibody and incubating, washing four times in buffer, applying enough biotinylated goat anti-polyvalent to cover tissue sections and incubating at room temperature, washing in buffer, applying streptavidin peroxidase and incubating at room temperature, adding chromogen/substrate and incubating, counterstaining, and finally dehydrating and using coverslips. The specificity and sensitivity of antigen detection was dependent on the specific primary antibody.

Results

Clinical and parasitological findings. All the test animals died due to the impossibility of recovery. The clinical manifestations of the affected animals included emaciation, anemia, exophthalmia, petechial conjunctivae, oral and nasal mucosae, as well as enlarged superficial lymph nodes. *T. annulata* piroplasms were identified in the erythrocytes of blood smears. The Giemsa-stained lymph node impression smears revealed the macroschizonts in mononuclear cells. Schizonts in the tissue sections of the lymph nodes were also detected by staining with modified Giemsa.

Necropsy findings. Following necropsy, the main observations included: edema and enlargement of lymph nodes; hemorrhages in lymph nodes and spleen; hemorrhages in subcutis and on most of the serous and mucous membranes; hemorrhagic endocardium and pericardium; severe jaundice in the subcutaneous fat and omentum; pale mucous membranes and petechial and ecchymotic hemorrhages in the mucosal and serosal surfaces; hemorrhages and volcanic and necrotic ulcers in the abomasum which rarely extended to the intestine; pulmonary edema and emphysema, together with ecchymotic hemorrhage on the lungs; enlargement of the liver, with ecchymotic hemorrhages on its surface; and white-spotted kidneys with hemorrhages on their surfaces.

Histopathological changes. In most cases, proliferative changes of varying degrees were observed in the lymph nodes leading to an increase in the number and size of follicles. Edema, extensive haemorrhages and proliferation of lymphocytes were seen on the surface and in the parenchyma. The peripheral and intermediate sinuses were dense and characterized by large numbers of lymphocytes and macrophages. In some cases, the involved lymph nodes had partially lost their natural structure, with fibrinous material penetrating into the cortical reticular framework (Fig. 1A).

Kidney revealed non-suppurative interstitial nephritis with infiltration of lymphocytes occasionally in the interstitial spaces of the cortex and medulla (Fig. 1B). Hemorrhage and lymphoblastic infiltration were observed in the portal, peri-portal and inter-lobule areas of the liver of affected animals. In the heart tissue, lymphoblastic infiltration foci were visible between muscle fibers. In the abomasum, there was severe cellular necrosis under the sub-mucosa, the muscular mucosa. These lymphocytes were associated with focal bleeding in the muscular areas.

Volcanic lesions, lymphoblastic infiltration and mitosis were present in both the mucosa and lamina propria. Severe lymphoblastic infiltration was also observed between the mucosa and sub-mucosa of the abomasum.

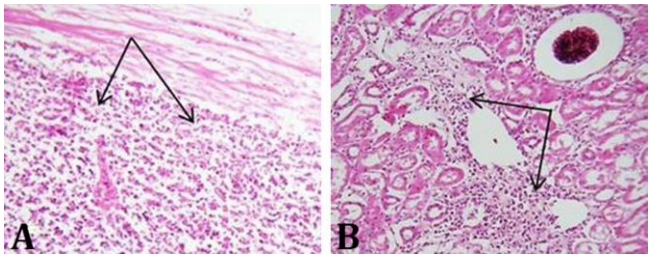

Fig. 1. Histopathological changes in infected cattle. **A)** Lymph nodes (focal proliferation of lymphocytes) and **B)** kidney. Arrows show the non-suppurative infiltration of lymphocytes, (H&E staining, 40×).

Immunohistochemistry. In this study, ab64261, a labeled streptavidin-biotin immuno-enzymatic antigen detection system was used. In almost all the observations, the cell population was composed of CD3 positive T-lymphocytes and macrophages labeled by MAC387, but not by CD20, and showed strong cytoplasmic staining. Most of the lymphocytes identified in lymph nodes, heart, liver, spleen, kidney, abomasum, and lung were CD3-positive T-cells. The IHC findings are summarized in Table 1 and the results of immunohistochemistry are shown in Figure 2. The results of the tissue PCR are shown in lymph nodes, liver, lung and abomasum (Fig. 3). The other samples, including heart, spleen and kidney were negative.

Table 1. Immunohistochemistry results for sections of the lymph node (LN), kidney (K), heart (H), spleen (S), liver (Li), lung (Lu), and abomasum (A) with primary antibodies anti-macrophages (MAC387), anti-CD20 and anti-CD3 T lymphocyte.

Antibody	Tissue type	Animal No.							
		1	2	3	4	5	6	7	8
MAC387	LN	+++	+++	+++	+++	+++	+++	+++	+++
	H	++	++	+	++	+++	+	--	--
	A	+++	+++	+++	+++	+++	++	+	++
	Li	++	++	++	+++	+	++	+++	+
	Lu	+++	+	++	--	+	++	+++	+
	S	--	--	--	--	--	--	--	--
	K	--	+	++	--	--	--	+	+
CD3	LN	+++	+++	+++	+++	+++	+++	+++	+++
	H	--	+	+	+++	+	+	+	+
	A	+++	+++	+++	+++	+++	+++	+++	+++
	Li	+	+	+	+++	+	--	--	+
	Lu	++	++	++	+++	+	++	+	++
	S	--	--	--	--	--	--	--	--
	K	+	+	+	+	+	+	+	+
CD20	LN	--	--	--	--	--	--	--	--
	H	--	--	--	--	--	--	--	--
	A	--	--	--	--	--	--	--	--
	Li	--	--	--	--	--	--	--	--
	Lu	--	--	--	--	--	--	--	--
	S	--	--	--	--	--	--	--	--
	K	--	--	--	--	--	--	--	--

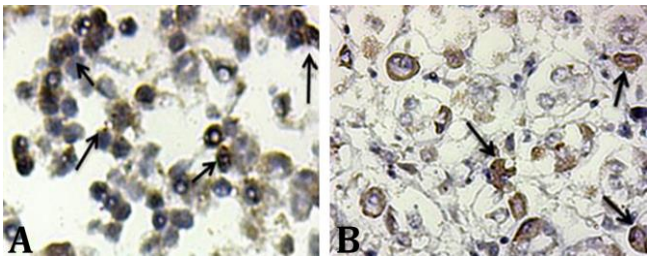

Fig. 2. Cells positive to MAC387 (mouse anti-human macrophages, streptavidin-biotin-peroxidase) in **A)** lymph nodes, and **B)** abomasum. Arrows show the positive cells, (IHC staining, 100×).

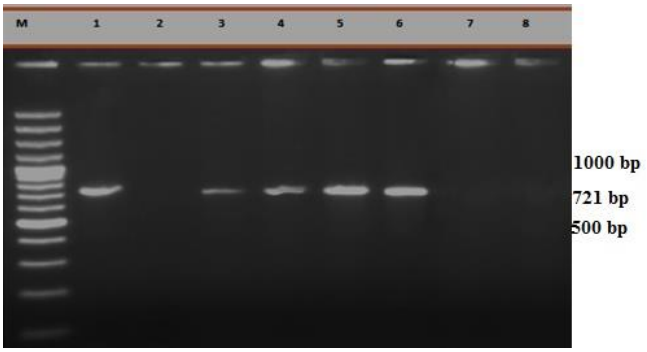

Fig. 3. The results of PCR. Lane M: Marker (100 - 1,000 bp), Lane 1: Positive control (721 bp), Lane 2: Negative control, Lanes 2-8: Test samples.

Discussion

As a tick-borne disease, bovine tropical theileriosis caused by *T. annulata* has high morbidity and mortality in cattle in Asia, North Africa and Southern Europe.[19] The disease is common in some parts of Iran and causes high mortality, particularly in exotic and cross-breed cattle.[20] In this study, all infected cattle showed symptoms of the acute and lethal theileriosis, including high fever, swollen lymph nodes, anemia, jaundice, and bleeding on the mucosa prior to death. Similar clinical signs of tropical theileriosis with high mortality have been reported in exotic breeds of cattle in Iran [12,21] and other countries.[6,22,23] The severity of clinical symptoms of the disease was dependent on the cattle breed, *Theileria* strain, and sporozoite inoculation rate.[24]

The macroscopic and microscopic pathological changes in the animals examined in this study resembled those reported for bovine tropical theileriosis.[11,25,26] The mononuclear cell infiltration with edema, congestion and hemorrhage were the prominent microscopic lesions in the abomasum, lymph nodes, liver, lungs, and heart, but the kidneys and spleen were rarely affected in this study.

The piroplasms of *T. annulata* were observed in erythrocytes and also schizonts in lymph node impression smears. It is very difficult to find small *Theileria* schizonts in tissue sections stained with H&E;[27] therefore, we used a modified Giemsa staining for tissue sections and the schizonts were successfully detected in lymph nodes.

Kirvar *et al*. used the 30.00 kDa merozoite surface protein gene as a target sequence by designing specific primers to amplify *T. annulata*.[28] The results of this study clearly demonstrated the high sensitivity and specificity of the designed PCR for detecting *T. annulata* species based on the Tams-1 gene for highly sensitive detection of the parasite.[29] The molecular examination confirmed *Theileria* infection in the lymph nodes, liver, lung and abomasum.

Immunohistochemistry was applied to study the population of mononuclear leukocytes in different tissues. The major cell population was composed of CD3 positive T lymphocytes and macrophages. The results of this study were in line with those observed in fetal bovine theileriosis in Portugal which identified T lymphocytes and macrophages, with only a few B lymphocytes present in different tissues of infected calves.[25]

Theileria annulata principally infects and transforms monocytes/macrophages and to a lesser extent B. cells.[29] The microscopic study of infected tissues and transformed schizont- infected cells indicated that the parasites enter myeloid cells of the monocyte/macrophage lineage.[27] The infected macrophages activate T lymphocytes in naïve cattle through a combination of cytokines and contact between T cells and infected cells.[27]

Many studies have shown that *Theileria*-infected mononuclear cells produce pro-inflammatory cytokines which are associated with clinical signs and pathological changes in different tissues, as seen in lethal tropical theileriosis.[30-32]

The results of a study indicated a high number of $CD4^+$ and $CD8^+$ lymphocytes during East Coast Fever (ECF) until the final stages of the disease.[33] The high number of these cells and macrophages in the lung indicated their role in the pathogenesis of the different stages of *Theileria parva* infection.[33] The presence of T lymphocytes in the lungs causes the secretion of cytokines leading to inflammation and edema of the lungs. Small amounts of B cells in the lymphocytic infiltrates of the lungs during the terminal stages of ECF suggests that the local humoral response is of little importance as is the case with other intracellular pathogens. Some researchers, in their study using immunohistochemistry entitled "ECF caused by *T. parva* is characterized by macrophage activation associated with vasculitis and respiratory failure", showed that parasitic lympho-proliferation leads to the activation of secondary and systemic macrophage syndrome which is similar to the results of this study.[16]

According to the findings of this study, it was deduced that the observed pathological alterations in the tissues of cattle with tropical theileriosis were caused by the expansion of schizont-infected macrophages, followed by intense stimulation and unrestrained multiplication of uninfected T lymphocytes leading to an efficient blockage of the specific immune response. This immune response deficiency may be responsible for the disease pathology. A more complete understanding of the immune response manipulation by *T. annulata* requires a thorough understanding of the molecular procedures related to *Theileria* which will apply to the control and activation of the gene expression profile in the infected leukocytes and ultimately the control and treatment of the disease.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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