

Pathological and molecular diagnostic study of theileriosis in cattle in Sulaimaniyah province, Iraq

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دراسة مرضية وتشخيصية جزيئية لمرض الثيليريا في الأبقار في محافظة السليمانية في العراق

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Summary

This study was undertaken with the aims of evaluating the efficiency of the PCR assay in investigation of the epidemiological status of tropical theileriosis in comparison with the classical microscopic examination of Giemsa-stained blood smear and detecting the gross and microscopic pathological changes in some organs of the infected animals. Blood and tissue samples were collected from 50 apparently healthy, tick-infested cattle during the period extended from the 1st of July to the 31st of August, 2008 in the modern slaughterhouse in Sulaimaniyah province. Blood smears were prepared from the blood samples and stained with Giemsa dye. The tissue samples were obtained from normal and lesion-presenting tissues of the lymph nodes, lungs and kidneys were investigated by the naked eye for the presence of any pathological changes. Following gross examination, the tissue samples, of the cattle that showed positive results for *Theileria annulata* parasites by PCR and Giemsa-stained blood smears, have processed for histopathological preparations.

When compared with microscopic examination of Giemsa-stained smears, the PCR assay detected a positive results in 8 (16%) of blood samples that revealed negative microscopy indicating that the PCR is more sensitive in diagnosis of tropical theileriosis. The gross pathological examination revealed lymph nodes enlargement, pulmonary congestion and edema and multiple pale areas in the kidneys. Microscopically, the principle pathological lesion was represented by a marked lymphoproliferative reaction within these organs.

Key words: *Theileria annulata*; Al Sulaimaniyah.

الخلاصة

انجزت هذه الدراسة لاجل تقييم كفاءة تقنية التفاعل المتسلسل لأنزيم البوليميريز (PCR assay) في الكشف عن الحالة الوبائية لمرض الثيليريا في الأبقار بالمقارنة مع طريقة الفحص المجهرى الروتيني لمسحات الدم الملونة بصبغة الكيمزا ولجل التحري عن التغيرات المرضية العيانية والمجهرية في بعض أعضاء جسم هذه الحيوانات. جمعت نماذج من الدم والأنسجة من 50 بقرة مصابة بالقراد في منطقة السليمانية خلال الفترة من 1 / 7 ولغاية 31 / 8 من العام 2008 لأجل دراسة التغيرات المرضية في العقد اللمفية والرئتين والكليتين في الأبقار المصابة بداء الثيليريا في الأبقار بواسطة الفحص العياني لتلك العينات. وبعد ذلك مررت النماذج النسيجية المأخوذة من الأبقار التي أظهرت نتائج موجبة للاصابة بواسطة تقنية التفاعل المتسلسل لأنزيم البوليميريز و صبغة الكيمزا للفحص المرضي النسيجي المجهرى.

بيّنت تقنية التفاعل المتسلسل لأنزيم البوليميريز وجود نتائج موجبة في 8 (16%) من عينات الدم التي أظهرت نتائج سالبة بالفحص المجهرى لمسحات الدم الملونة بصبغة الكيمزا وهذا يدل على أن هذه التقنية أكثر دقة في تشخيص داء الثيليريا في الأبقار. بين الفحص المرضي العياني للأبقار المصابة تضخم العقد اللمفية واحتقان

وخزب الرنتين وظهور باحات شاحبة متفرقة في الكليتين. مجهرياً تَمَثَّلَت الافة المرضية الرئيسية في وجود تفاعل تكاثري لمفاوي واضح المعالم marked lymphoproliferative reaction في هذه الأعضاء.

Introduction

Pathology of tropical theileriosis involves rapid, massive, uncontrolled proliferation of infected leukocytes & macrophages and tissue destruction observed during infection with *T. annulata* schizonts due to the nature of the disease induced by *Theileria* parasites “a lymphoproliferative disease characterized by non-specific T lymphocyte proliferation” (1-4) resulting in enlargement of the draining lymph nodes due to proliferation of both infected and non-infected T-cells (5). Microscopical lesions are characterized by proliferating lymphoblastoid cells and necrosis in lymphoid organs, lungs, liver, kidneys, the gastrointestinal tract and other tissues (6). In Iraq, tropical theileriosis poses a big challenge to the cattle industry in Iraq (7), however there is an insufficient epizootiological knowledge regarding this important disease. The diagnosis of theileriosis is usually based on identification of the piroplasm and schizont stages of the parasite in Giemsa-stained blood and lymph node smears (8). The present study was undertaken with the aims of evaluating the efficiency of the PCR assay in investigation of the epidemiological status of tropical theileriosis in comparison with the classical microscopic examination of Giemsa-stained blood smear and detecting gross and microscopic pathological changes in some organs of the infected animals.

Materials and Methods

Study area and animal sampling

The blood and tissue samples of the present study were collected from 50 apparently healthy, tick-infested cattle during the period extended from the 1st of July to the 31st of August, 2008 in the modern slaughterhouse in Sulaimaniyah province. A five milliliters blood sample was withdrawn from the jugular vein of each animal by a 5 ml disposable syringe with 18-gauged needle. The blood then was emptied into 2 commercially prepared EDTA-containing tubes, kept in an insulated container with ice packs and brought to the laboratory. Giemsa-stained blood smears were prepared from these blood samples and examined microscopically by the X 1000 magnification power for the presence of *Theileria* parasites. The remaining quantities of the blood samples were stored at -20 °C until used for DNA extraction.

Processing of blood samples for PCR assay

DNA was extracted from each blood sample using a genomic DNA extraction kit (Promega, USA) according to the manufacturer's instructions.

Theileria annulata specific primers (N516 / N517 primers) derived from the gene encoding the 30-kDa major surface antigen of *T. annulata* merozoite was used in the amplification reaction under the conditions described by d'Oliveira *et al.* (9). Briefly, PCR was performed in a final reaction volume of 100 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.1% Tween 20, deoxynucleoside triphosphate (200 µM each), 2.5 U of *Taq* DNA polymerase (Peqlab Biotechnologie, Erlangen, Germany), forward and reverse primers (160 pmol each) and 5 µl of the processed DNA sample. The PCR reaction (performed in an automatic DNA thermal cycler, Eppendorf, Germany) involved an initial denaturation step at 94 °C for 3 minutes; 30 thermal cycles, each of which consisted of a denaturation step of 1 minute at 94 °C, an annealing step of 1 minute at 55 °C, and an extension step of 1 minute at 72 °C; a final extension step of 5 minute at 72 °C and a holding step at 4 °C until the samples were taken out from the thermal cycler. Amplified PCR products were separated by electrophoresis on 1% agarose gel and were subsequently visualized and photographed using an ultraviolet trans-illuminator.

Histopathological examination

Tissue samples were obtained from normal and lesion-presenting tissues of the lungs, kidneys and lymph nodes. Tissue samples obtained were investigated by the naked eye for the

presence of any abnormalities related to their morphology, size, color and consistency. In addition, external and/or cut surface lesions were described regarding their location, distribution (focal or multifocal), size, shape, color and texture. Following gross examination, the samples were kept in 10% formalin for 24-48 hours. The formalin-fixed tissue samples, of the cattle that showed positive results for *Theileria annulata* parasites by Giemsa-stained blood smears and/or PCR, have undergone a series of histopathological preparations according to (10) and glass slide tissue sections were stained by routine (hematoxylin and eosin) and Giemsa stains and examined by different magnifying powers of light microscopy for the presence and detailed description of *Theileria annulata* schizonts and any noticeable lesions.

Results

Morphology of *Theileria annulata* parasites in Giemsa-stained blood smears

The microscopic examination of the Giemsa-stained blood smears of infected cattle revealed the presence of free and intracellular forms morphologically compatible with theilerial piroplasms and schizonts. Infected erythrocytes showed morphological disorders represented by round-shaped appearance and irregular thorn-like protrusions. The piroplasms within their cytoplasm were predominantly round or oval in shape (Figure 1) but rod and comma forms have also been identified. They were observed as individual (one piroplasm per erythrocyte), double, triple and tetra-forms.

The schizonts were observed either as free forms or as intracellular forms in some of the monocytes and lymphocytes within the blood smears. They appeared as circular or irregularly shaped structures with blue cytoplasm and varied numbers of red chromatin granules (Figure 2).

Polymerase chain reaction assay

The PCR assay detected a positive result to Mediterranean theileriosis in 35 (70 %) of blood samples out of the total 50 samples involved in the present study (Figure 3) whereas the microscopical examination of Giemsa-stained blood smears detected a positive result in only 27 (54 %) of the blood samples.

Pathological findings

The postmortem examination of cattle clinically infected with *T. annulata* parasites revealed lymph nodes enlargement associated with edema and hemorrhage (Figures 4) involving particularly the draining ones (lymph nodes of the prescapular and prefemoral regions as well as those of the supramammary region in females). Microscopic examination of routinely-stained tissue sections of the affected lymph nodes showed reticulo-endothelial hyperplasia, reactive follicular hyperplasia, marked enlargement of germinal centers and slight increase of the interfollicular lymphoid tissue within the cortical and paracortical regions (Figure 5). In addition, microscopical examination of Giemsa-stained tissue sections of the affected lymph nodes showed the presence of different sized and shaped schizont forms of *Theileria annulata* parasites either extracellularly or intracellularly within many of the lymphocytes and sinusoidal macrophages (Figure 6).

In addition, the gross pathological examination showed variable extents of pulmonary congestion, hemorrhage, edema and emphysema. Microscopically, these lesions were indicated by distinct distension of the pulmonary blood vessels with RBCs, presence of proteinaceous fluid within the alveolar spaces, occurrence of wide emphysematous areas (alveolar and interstitial emphysema) and infiltration of mononuclear inflammatory cells, mainly lymphocytes within the interstitial tissue of the lung (Figure 7).

The kidneys of infected cattle display variable sized, pale areas (pseudoinfarct areas) distributed over their entire external surfaces (Figure 8) and within their parenchyma. The histopathological examination of these kidneys exhibited multifocal areas of marked interstitial nephritis represented by an extensive lymphocytic infiltration within the interstitial tissue of the renal parenchyma (Figure 9).

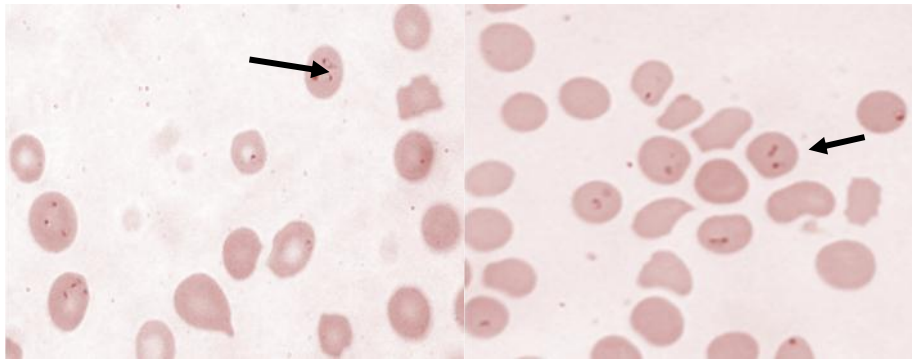


Figure 1: Giemsa-stained blood smears of infected cattle reveal the presence of intra-erythrocytic forms (arrows) morphologically compatible with theilerial piroplasms. Some of the infected erythrocytes showed morphological disorders represented by round-shaped appearance and irregular thorn-like protrusions (X 1000).

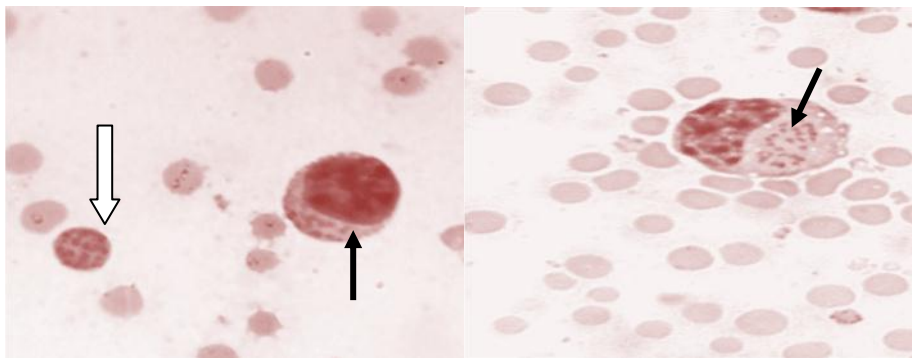


Figure 2: Giemsa-stained blood smears of infected cattle reveal the presence of extracellular (white arrow) and intracellular (black arrows) schizont forms of *Theileria annulata* parasites. Intra-erythrocytic piroplasm forms are also evident (X 1000).

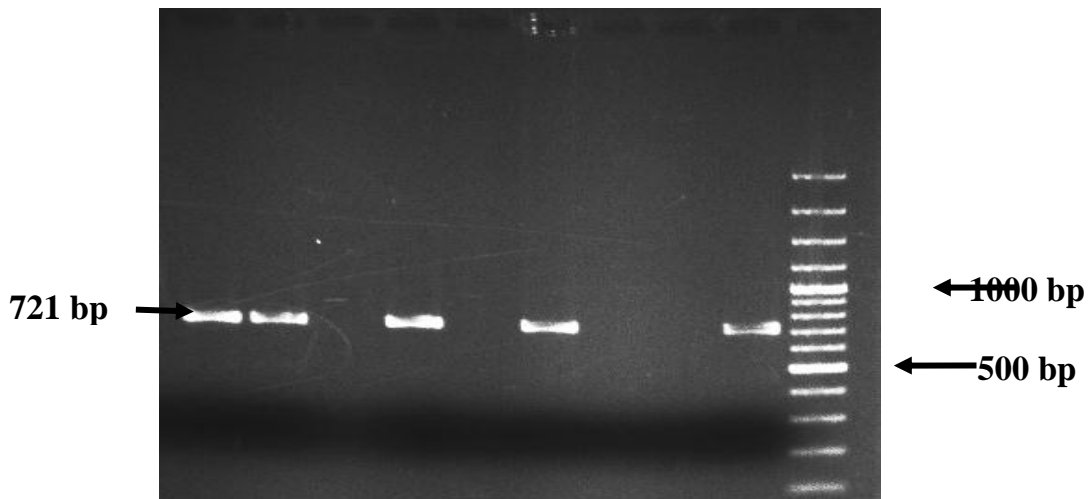


Figure 3: Detection of *T. annulata* in blood samples by the PCR assay. The figure shows results of agarose gel electrophoresis of amplified DNA extracted from blood samples obtained in the present study. Lanes 1, 2, 4 and 6 infected blood samples; lanes 3, 5 and 7 non-infected blood samples; lanes 8 & 9 controls negative & positive respectively and lane 10 molecular size marker.

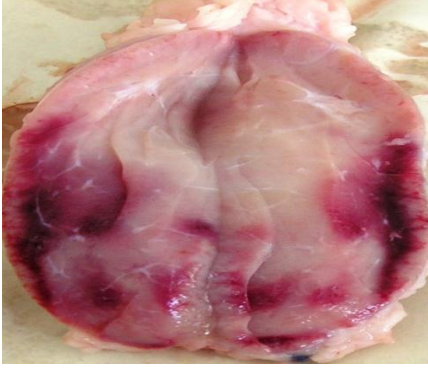


Figure 4: Longitudinal section of a prefemoral lymph node obtained from a cow infected with *Theileria annulata* parasites. It appears edematous and hemorrhagic.

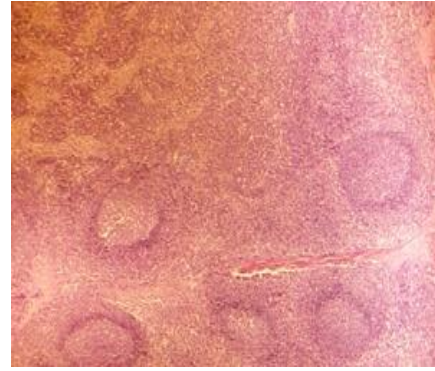


Figure 5: Microscopic view of the lymph node illustrated in figure 3 showing reactive follicular hyperplasia in the cortex, enlargement of germinal centers and slight increase of the inter-follicular lymphoid tissue. H and E stain, X100.

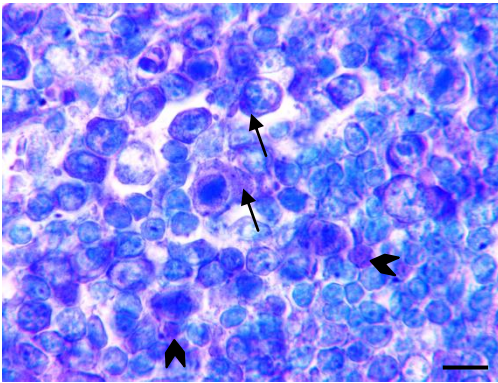


Figure 6: Microscopic view of a lymph node tissue section of a cow infected with *Theileria annulata* parasites shows the presence of schizont forms of the parasites either extracellularly (arrow heads) or intracellularly (arrows) within many of the lymphocytes and the sinusoidal macrophages. Giemsa stain, size bar = 20 µm.

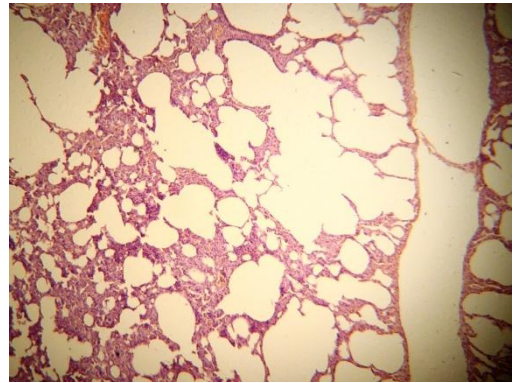


Figure 7: Microscopic view of a cow lung infected with *Theileria annulata* parasites showing marked interstitial and alveolar emphysema associated with thickening of interalveolar septa. H and E stain, X100.

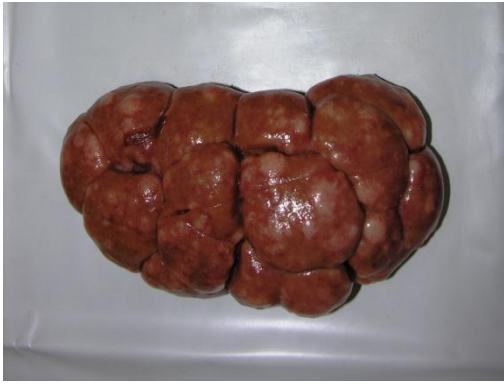


Figure 8: kidney of a cow infected with *Theileria annulata* parasites. Variable sized, pale white areas are seen on the cortical surface.

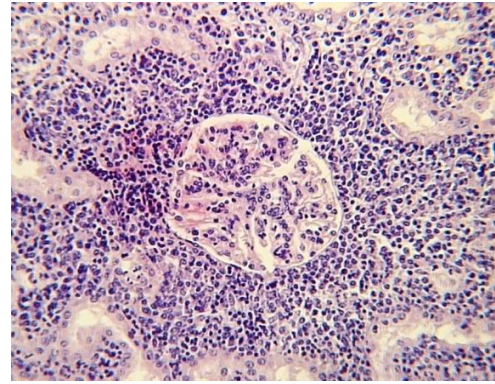


Figure 9: Microscopic view of the kidney illustrated in figure 8. Marked interstitial nephritis is apparent as indicated by the extensive infiltration of mononuclear inflammatory cells (mainly lymphocytes) within the interstitial tissue. H and E stain, X 400.

Discussion

Giemsa-stained blood smears revealed that cattle erythrocytes infected with the piroplasm forms of *Theileria annulata* parasites showed round-shaped appearance and irregular thorn-like protrusions. These erythrocytic morphologic disorders are attributed to presence of the parasites in the erythrocytes, erythrocyte oxidation and immune-mediated processes (11-13) and they are generally in agreement with findings of (14). An interesting finding within these Giemsa-stained blood smears was the observation of intracellular (in some of the monocytes and lymphocytes) and extracellular schizont forms of *Theileria annulata* parasites. To date, there is no precise explanation for this finding (the presence of *Theileria annulata* schizonts outside the host cells), however it may be hypothesized that this finding is attributed to an apoptosis-related mechanism, as it has been described for the malaria parasite by which, the parasites induce host cell apoptosis causing the liberation of the parasite from the host cell and its extracellular localization (15). This result is generally in agreement with that of (16) who pointed out that most of the schizont forms of *Theileria* spp. (China) of sheep and goats are located outside the host cells.

The PCR assay detected the DNA of *Theileria annulata* piroplasms in 8 (16%) of cattle blood samples with negative microscopy. This result indicates that the PCR assay is more sensitive than parasite detection by light microscopy of Giemsa-stained blood smears. This finding is generally compatible with findings of other authors (17-19). The low sensitivity of light microscopy of Giemsa-stained blood smears is ascribed to the fact that this method is dependable for the detection of acute cases but has limited value for chronic and long-lasting carrier cases, where only low numbers of *Theileria* piroplasms exist (20).

The gross and microscopic pathological changes observed in cattle infected with *Theileria annulata* parasites are generally compatible with pathological lesions reported by other authors (6, 21, 22, 23, 24). They include lymph nodes enlargement involving particularly the draining ones. Microscopic examination of the affected lymph nodes showed reticulo-endothelial hyperplasia and reactive follicular hyperplasia. The possible

explanation for this finding is the nature of the disease induced by these parasites (a lymphoproliferative disease) and its outcome represented by enlargement of the draining lymph nodes particularly in its early phases (25). Another possible explanation for this finding is the fact that cells infected by *theileria annulata* schizonts induce non-specific T lymphocyte proliferation (4) resulting in enlargement of the draining lymph nodes due to proliferation of both infected cells and non-infected T-cells (5). These two possible explanations for the development of this finding are supported by the microscopical examination of Giemsa-stained tissue sections which showed the schizont forms of *Theileria annulata* parasites within many lymphocytes and sinusoidal macrophages of the affected lymph nodes.

Variable extents of pulmonary congestion, hemorrhage, edema and emphysema were also observed upon gross pathological examination of cattle clinically infected with *Theileria annulata* parasites. Microscopically, these lesions were indicated by distinct distension of the pulmonary blood vessels with RBCs, presence of proteinaceous fluid within the alveolar spaces, occurrence of wide emphysematous areas and infiltration of mononuclear inflammatory cells, mainly lymphocytes within the interstitial tissue of the lung. These pathological changes are ascribed to the fact that cells infected with *Theileria annulata* schizonts induce polyclonal naïve T lymphocyte proliferation (4) which switches to a T helper 1 phenotype, producing large quantities of IFN- γ (5). It is believed that these abnormally high levels of IFN- γ together with excessive production of pro-inflammatory cytokines (including IL-1 α , IL-1 β , IL-6 and TNF- α) are probably the main causes of parasite induced pathological lesions (26, 27).

In addition, the gross pathological examination revealed distribution of variable sized, pale white areas over the entire external surfaces and within the parenchyma of the kidneys. These areas which are sometimes referred to as “pseudo-infarcts” (17) are probably attributed to infiltration of the renal interstitial tissue with large numbers of mononuclear inflammatory cells (mainly lymphocytes) due to specific and non-specific uncontrolled T lymphocyte proliferation induced by *Theileria annulata* schizonts (1, 4, 28).

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