Volume 5, issue 12, 2016 ● e-ISSN: 1857-8187 ● p-ISSN: 1857-8179

Research Article

Tularemia under Histological and Immunohistochemistry Examinations

Animal Biology

Keywords: Tularemia, Francisellatularensis, Lepus europaeus, tissue samples.

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Abstract

Francisella tularensis is the aetiological agent of tularemia. Hares, rabbits, and small rodents are the main hosts. Humans can be infected and develop severe clinical symptoms. The occurrence of tularemia was studied with histological and immunohistochemistry examinations in257 Lepus europaeus tissue samples in different parts of Macedonia (FYROM). Francisella tularensis seems to be a important pathogen inthe surveyed area.The postmortem findings in hares dying of tularemia were characterized by focal coagulative necrosis in liver, spleen and bone marrow, with high numbers of grampositivebacteria for Francisellatularensis. In Lepus europaeus tissue samples the most characteristic findings were hemorrhagic enteritis and typhlitis, although necrotic lesions could occur in liver, spleen and bone marrow. Presence of granulomatous inflammation was used as an indicator of Francisellatularensis infection. Further samples are being analyzed and advanced tests will be used in order to identify the most proper and affordable screening and confirmatory tests for monitoring the epidemiological situation of Francisellatularensisinfection. Sampling of tissues and organs is a simple process, less costly and more effective for the evaluation of cases of tularemia in the population of rabbits under histological and immunohistochemistry examinations.Postmortem examination ashistological and immunohistochemicalexamination were be simplesthe method of work.

1. Introduction

Tularemia is a serious infectious disease caused by the intracellular bacterium *Francisella tularensis*. It causes fever, and sometimes ulceration at the site of entry and/or swelling of nearby lymph nodes. It can cause severe pneumonia. *Francisella tularensis*is a bacteria which affects both humans and animals. Humans can become infected through several routes, including tick and deer fly bites, skin contact with infected animals, ingestion of contaminated water, inhalation of contaminated aerosols or agricultural dusts and laboratory exposure.*Francisella tularensis* is a pathogenic species of Gram-negative, rod-shaped coccobacillus, an aerobe bacterium. It is non-spore forming, non-motile and the causative agent of tularemia, the pneumonic form of which is often lethal without treatment. Rabbits, hares, and rodents are especially susceptible and often die in large numbers during outbreaks.

Symptoms vary depending on the route of infection. Although tularemia can be life-threatening, most infections can be treated successfully with antibiotics. Steps to prevent tularemia include use of insect repellent, wearing gloves when handling sick or dead animals and avoiding mowing over dead animals. Caseating granulomata with or without multinucleated giant cells develops in some lesions.*Francisella tularensis*as a facultative intracellular bacterium infect most cell types, but primarily infects macrophages in the host organism. Entry into the macrophage occurs by phagocytosis and the bacterium is sequestered from the interior of the infected cell by a phagosome. *Francisella tularensis* then breaks out of this phagosome into the cytosol and rapidly proliferates. Eventually, the infected cell undergoes apoptosis, and the progeny bacteria are released to initiate new rounds of infection.Early tularemic lesions may demonstrate areas of focal

necrosis surrounded by neutrophils and macrophages. Later, the necrotic areas become surrounded by epithelioid cells and lymphocytes. Recently, there are reported severe human cases in Kosovo, Montenegro, Macedonia and Albania. The European brown hare (*Lepus europaeus*) plays an important role as reservoirs of *Francisella tularensis* infection.*Lepus europaeus*in Europe is widely distributed and as a game specieshas widely been introduced to countries across the globe from sea level to 2,300 m.

2. Materialsand methods

To realize the target of *the* study for*Francisella tularensis* infection estimates under histological and immunohistochemistry examinations we realisated a full study in *Lepus europaeus*tissue samples.In this context, all estimates are basedon histopathological andimmunohistochemistryresults. Samples represented*Lepus europaeus*tissue samples collected and evaluated during 2014-2015 and Spring-Autumn 2016 time period. Samples are taken from different villages as Debresh, Nerove, Allbance, Presille, Bellushine, Haracine, Tearce in Macedonia (FYROM).

Postmortem Examination. European brown hares, shot at different locations in Macedonia (FYROM)during several hunting events over two winter hunting seasons (2014–2015 and 2015–2016), were screened by the slide agglutination test using stained bacteria (Bioveta Inc,) and whole blood. Carcasses of two deads adult male *Lepus europaeus* submitted for histopathological andimmunohistochemistry. At the same time 257tissue samples of *Lepus europaeus* (hunting seasons) were collected and examinated. All histological sammples were necropsied under appropriate biosafety conditions at the Vet lab in Macedonia (FYROM). Tissue sampleswere categorized as same-year juveniles and older, based on the so-called Stroh mark. The body condition was estimated with a simplified categorical (good, moderate, weak) version of the kidney fat index. Tissue samples were collected for histology are fixed in 10% buffered formaline. The tissue samples *Lepus europaeus*negative for *Francisella tularensis* infectionused as negative controlsand comparative samples. Four micron-thick sections of formaline-fixed, paraffine-embedded tissue samples were stained with hematoxylin and eosine and examined by light microscopy.Immunohistochemistry examinations (IHC) was applied for the demonstration of *Francisella tularensis* lipopolysaccharide antigen in tissue sections. Briefly, after deparaffinization and antigen retrieval (in a microwave oven at 750 W for 20 minutes in citrate buffer pH 6.0), the sections were incubated in 3% H_2O_2 solution for 10 minutes and then in a 2% solution of skimmed milk powder for 20 minutes. The samples were incubated overnight at 37^oC with a 1:6.000 dilution of a *F. tularensis* lipopolysaccharide specific mouse monoclonal antibody (clones FB11 and T14, MAB8267, Chemicon International Inc, Southhampton, UK). Antibody binding was detected by a horseradish peroxidase-labeled polymer (EnVisionþ Kit, Dako, Glostrup, Denmark). A serial section incubated with phosphate buffer solution was used as a negative control.

3. Results and discussion

Histopathological examinations from 257 issue samples of *Lepus europaeus* at the Vet lab in Macedonia (FYROM), in a considerable number of samples resulted with gross pathological lesions in lung, pericard, kidney, testicle, bone marrow and liver.

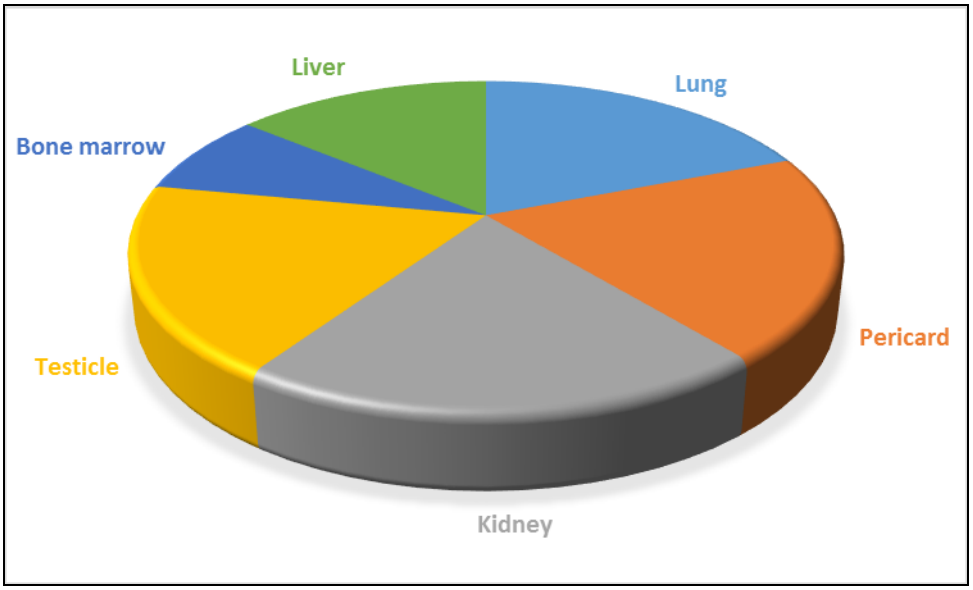

Figure 1.Cases with gross pathological lesions in the organs.

All carcasses were necropsied under appropriate biosafety conditions at the Vet lab in Macedonia (FYROM)were categorized as same-year juveniles and older, based on the so-called Stroh mark. Immunohistochemical examination used based in *F tularensis* antigen was detected in all cases presenting tissue lesions.

Figure 2. Sampling in rabbits.

The bacterial antigen revealed granular or amorphous staining and was clearly associated with histological lesions. In several cases, *F.tularensis* antigen occurred in small quantities or was absent in some areas of granulomatous inflammation. In all cases, the bacterial antigen was

observed in large amounts, mostly extracellularly, in foci of tissue necrosis. Intracellular *F.tularensis*was found in macrophages and giant cells in the majority of cases and less frequently in other cell types, such as tubular epithelial cells of the kidney, testis, and epididymis, epithelial cells of the renal pelvis, hepatocytes and bronchiolar epithelial cells. Extracellular and intracellular labeling was present in the inflammatory exudate situated in the lumen of airways, renal tubuli and pelvis and tubuli of the testis and epididymis. Bacterial antigen was visualized as fine intracellular granular structure within intact alveolar epithelial cells, hepatocytes, and intravascular macrophageswhereas extracellular labeling was present in blood vessels. Under histological examination we identified by pathological observation coalescing granulomatous inflammation, which completely replaced the normal tissue structure of the affected organs. After that, the foci were randomly distributed in the organs and serosal membranes were frequently involved. Macrophages were the dominant constituent cell type, but other cells were found occasionally including lymphocytes, heterophil granulocytes, multinucleated giant cells and fibrocytes as show in figure 2. Granulomatous inflammation was found with microscopic examination but not with gross pathological examination in the mediastinal lymph nodes. In several cases the coalescing granulomatous inflammation in the lungs contained no or only minor necrotic areas. Foci of granulomatous inflammation with central necrosis were found in the liver, bone marrow, mammary gland, spleen, and mediastinal lymph nodes.The postmortem findings in hares dying of tularemia in the autumn were characterized by focal coagulative necrosis in liver, spleen and bone marrow, with high numbers of bacteria. In hares dying during winter months, the most characteristic findings were hemorrhagic enteritis and typhlitis, although necrotic lesions could occur in liver, spleen and bone marrow.

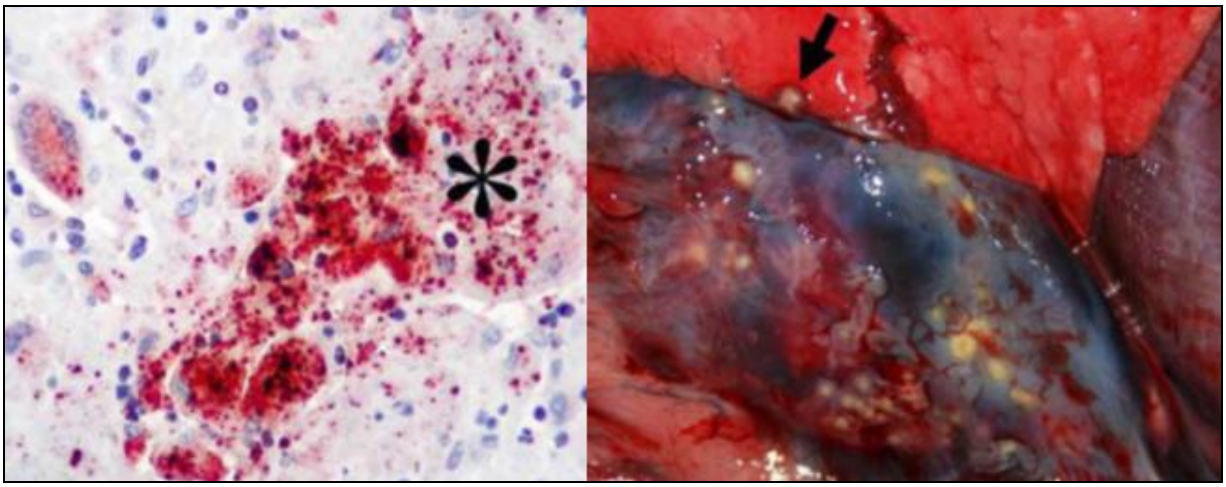

Figure 3. Lungand pericardium lesions from tularemia in*Lepus europaeus.*

This study shows except the presence of Tularemia in European Brown hares in Macedonia (FYROM) and the value and efficiency of alternative usedmethods asimmunohistochemical and histological examination. So tularemia is present in the population of European brown hare in Macedonia(FYROM). Serological methods are used for the diagnosis of tularemia in humansand they can be applied in animals. But serology examinations has limited use in highly susceptible

species of animalswhich usually die before specific antibodies develop.Additionally serological examinations always require blood sampling or fluid which is conceived as a complex process in the event of death of the host.While sampling of tissues and organs is a simple process, less costly and more effective for the evaluation of cases of tularemia in the population of rabbitsunder histological and immunohistochemistry examinations. The study serves as a good basis to realize other achievements as well.This research can be extended in all the territory of the Macedonian state,but also elsewhere in the border states and borders between them. It covers the areas near the of Kosovoborder in which Tularemia has been present about at least 10 years ago and are also plenty of cases in humans. The study can also be used as a good basis in order to run other studies in the whole population of lagomorphsand the dynamics of this population in all the territory of the Macedonian state.

4. Conclusions

Tularemia presence in the population of European brown hare(*Lepus europaeus*) in Macedonia(FYROM)can be clearly evidenced with histological and immunohistochemistry examinations. Under this examinations resulted with gross pathological lesions in lung 38 samples, pericard 40 samples, kidney 41 samples, testicle 37 samples, bone marrow 16 sample and liver 28 issue samples.Histological and immunohistochemistry examinationsbesides enabling simplicity and low cost of examining the sustainable positivity value especially in cases of death of the rabbits, when sampling fluids risk of infection except fluid for serological tests become more difficult over time of death that lagomorphs. This becose serological methods are used ferology has limited use in highly susceptible species of animals, which usually die before specific antibodies develop. The study serves as a good basis to realize other achievements as well. It covers the areas near the border of Kosovo, in which Tularemia has been present years ago and with difficult cases and in humans. The study can also be used as a good basis in order to run other studies in the whole population of lagomorphs in all teritory of Macedonian state and boders between them and Kosovo.

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