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Tularemia's Data Monitoring in *Lepus europaeus* of Macedonia

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Abstract:

This article provides data for the study conducted by the above group of researchers in the monitoring of tularemia in the wild rabbit populations in the western part of Macedonia (FYROM). This study was conducted in 2014-2017. The European brown hare (Lepus europaeus) plays an important role as reservoirs of Francisella tularensis infection. Samples are taken from different villages of Macedonia such as: Debresh, Nerove, Allbance, Presille, Bellushine, Haracine, Tearce . etc. In order to study the prevalence of Francisella tularensis infection we designed a longitudinal study in hares based on both agglutination test and histopathological results. During the two years, 280 blood samples were collected from the wild rabbit

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population which were subjected to the serological test, resulting in a total of 27 positive samples for tularemia presence. Out of these samples were internal organs, such as kidneys, melts, spleen, lungs, testicles, and bone marrow. These samples were evaluated for pathological changes and possible lesions. The results obtained are reflected in this article. Partial results of the study were presented and published in several regional scientific journals. The study of tularemia in the wild rabbit population in this region is the first of its kind and was conducted in terms of a doctoral study. It gives a very important information on this zoonotic disease and has value not only in veterinary but also in public health.

Key words: *Francisella tularensis* infection, *Lepus europaeus,* samples, Macedonia.

INTRODUCTION

Tularemia is a zoonotic disease which exists endemically in most European countries (1, 2, 4). Even in endemic foci, the occurrence of tularemia varies widely between regions and tularemia may emerge annually within a 5-year period or may be inactive for more than a decade, for yet unknown reasons (3, 5). The re-emergence of tularemia has been reported in several European countries, In Europe, this notifiable infection is caused by *Francisella (F.) tularemia* subsp. *holarctica* (type B), in contrast to the highly pathogenic *F. tularensis* subsp. *tularensis* (type A), which is mostly found in the USA (6). In Europe, tularemia is most frequently seen in hares (*Lepus* spp.) and other small animals such as rabbits, which serve as reservoir hosts and are linked to enzootic transmission (7). Arthropods (ticks, mosquitoes and biting flies) are relevant vectors and may serve as a long-term reservoir (8). Tularemia is transmitted to humans by direct contact with infected animals, through contaminated water or food, or by vectors such as mosquitoes or ticks (9). In hares and rabbits, tularemia usually causes an acute and fatal septicemia, however sometimes shows

a sub acute to chronic disease course (10). The Western Balkans is the centere of the Tularemias presence in the wild hamsters and cases are also exposed to people (11) The European brown hare is reported to be a common reservoir of tularemia, and its public health importance is accentuated by being one of the most significant European game species (2, 9, 12). Despite the significant role of these lagomorphs in the ecology of tularemia and its importance as a human infection source, no report has been published so far about the pathological and microbiological examination of naturally infected European brown hares (4, 7, 8). The aim of the present study was to identify the gross and histological lesions characteristic for *F tularensis* infection of European brown hares (9, 13). This essential information can make it easy for both the detection of tularemia in brown hares by hunters and the recognition and identification of this dangerous zoonotic disease by diagnosticians (12).

METHOD

Animals

This study will include a rabbit (*Lepus europaeus*) hunted in various areas of the Republic of Macedonia during the two winter hunting seasons (2014-2015 and 2015-2016).

Tissues

Blood. From captured or killed rabbits, blood will be taken, from which serum will be separated centrifuged at 3000 rpm for 5-10 minutes. Separated serums can be analyzed directly or stored at -200C until analysis.

Tissues and organs: Seronegative rabbit tissue samples will serve as a negative control. All carcasses will be evaluated under adequate biosecurity conditions at the Veterinary Laboratory. Based on kidney fat, the carcasses will be evaluated in three categories (moderate moderate good). The tissue samples will be collected fresh (for microbiological examination) and stored in 10% buffer formaldehyde for

histological examination. The organs to be accumulated are: heart, pericardium, lungs, liver, spleen, kidney, small intestine, and bone marrow, tests and epididymis, ovary glandular glands, mediastinal lymph nodes. Prefabricated tissue blocks will be prepared from fixed tissues in formalin. From them microscopic strings will be prepared which will be stained with the classic hematoxylin eosin and ABC method. The prepared strings will be examined in a light microscope.

Serological method. The collected samples will be analyzed with the agglutination assay using the pre-colored hematoxylin (blue) antigen. Samples of wild rabbits that will result positive will be subjected to further laboratory examinations.

Immunohistochemistry of Avidin Biotin Complex (IHC-ABC).

Immunohistochemical Method (IHC) Avidin Biotin Complex (ABC), IHC-ABC, will be applied for demonstrating the lipopolysaccharide antigen of *F tularensis* in the tissue sections. Immediately after deparafining and antigen extraction (in a microwave oven at 750 W for 20 minutes in citrate buffer, pH 6.0), the incubation will be incubated in 3% H2O2 solution for 10 minutes at pH 6.0 and then in one 2% skim milk solution for 20 minutes. Samples will be incubated overnight at 370 C with a 1: 6000 dilution of *F tularensis*. Lipopolysaccharide with specific monoclonal antibodies produced in mice (clones FB11 and T14, MAB8267, Chemicon International Inc., Southhampton, UK). is detected by the peroxidation reaction and the use of specific substrate, a dark brown-brown reaction (EnVisionb Kit, Dako, Glostrup, Denmark). A series of cuts will be incubated with buffic phosphate solution and will be used for negative control. Use immunohistochemical avidin-biotin complex (IMHC -ABC) method to be performed on fixed tissue in formalin by observing the accompanying kit protocol.

Statistical Methods

The obtained results will be processed using statistical methods STAT and R.

RESULTS

Table 1 gives the sample data obtained during the study period. The obtained results are provided by the application of the serological method.

Table 1. Data obtained during the study period.

No	Period of samples collected	Nr of Samples	Negative samples	Positive samples
1	2014-2015	34	32	2
2	2015-2016	53	50	3
3	2016-2017	193	171	22
3	**Total**	**280**	**113**	**27**

Table 2. Cases With Gross Pathological Lesions in the Organs of Seropositive European brown hares.

No	Organs	Number of case with gross Pathological lesions	Number of case with no gross Pathological lesions
1	Lung	13	14
2	Pericardia	10	17
3	Kidneys	16	11
4	Testicles	7	7
5	Bone marrow	4	23
7	Lymph node	19	8
6	Liver	8	19
	Total (27)		

The foci identified by pathological examination corresponded to focal or 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 serosa 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 fibrocystic as shown in figure 2. Granulomatous inflammation was found

with microscopic examination but not with gross pathological examination in the meditational lymph nodes (7). 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 meditational lymph nodes (1, 13).

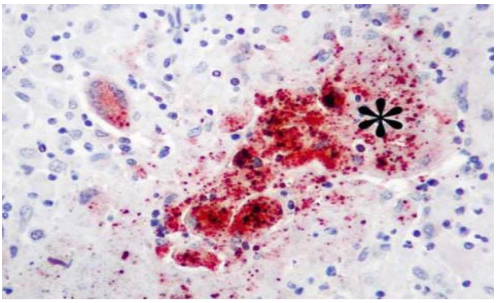

Figure 1. Lung of *Lepus europaeus positive* for tularemia.

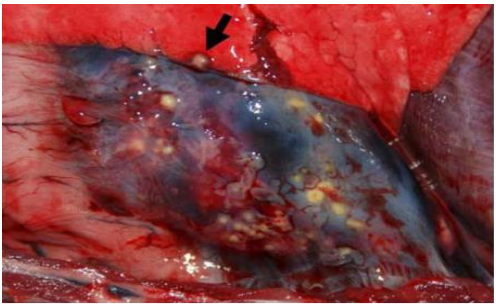

Figure 2. Pericardium and lung of *Lepus europaeus* positive for tularemia.

CONCLUSIONS

The study is the first of its kind to be carried out in the wild rabbit population of Macedonia (FYROM). It resulted in the identification of Tularemia in the wild rabbit population. This study is a confirmation of tularemia cases in areas bordering Macedonia and Kosovo.

Serological methods, Immunohistochemical (IHC) Avidin [Biotin Complex (ABC), IHC - ABC and histological examination have been applied for the identification of tularemia.

The results obtained from this study are consistent with the data obtained from the Research Institutions in Macedonia as well as the neighboring countries. The study provides a good basis for wider national and regional studies.

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