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CHECK OF WILD RABBIT MEAT IN MACEDONIA

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ABSTRACT

In this duty were performed the immunohistochemical examinations of 295 tissues and organs of suspected rabbits and 70 specimens from the tissues of the rabbits killed by the hunters in Macedonia in the regions of Dibresh, Nerove, Allbance, Presille, Bellushine, Haracine, Tearce etc.. These samples were collected from the shops and the restaurants which served rabbit meat in theor menu. The number of collected samples was considerable considering that the population of the above mentioned areas prefers mostly the consumation of the wild rabbit meat. The meat of wild rabbit is consumed by the hunters and their families as well as some luxurious restaurants. The meat of wild rabbit in Macedonia is used also in touristic areas. The collected samples were sent at the SKE Lab at the Faculty of Veterinary Medicine in which were realized 70 formalin-fixed samples according to the protocol which accompanied the Kit. Based on the fat of the kidneys, the carcasses were analyzed in three categories (good, moderated weak) The tissue samples were collected to be examined fresh or stored in 10% buffered formalin for histological and immunohistochemical examination. From the organ samles were prepared microscopic stripes that were stained with the classic hematoxylin eosin and ABC method. The prepared stripes were examined in a light microscope laboratory. Immunohistochemistry (IHC) model Avidin Biotin Complex (ABC), IHC-ABC, was applied and detected by microscopy the presence of F-tularensis lipopolysaccharide antigens in the rabbit tissue sections. Positive with a lesion in the organ resulted 12 samples (0.6%) of the examined tissues with immunohistochemistry were found to be be affected at least one of the observed organs. The total of the immunohistologic positive samples was dominated by the lung with 5 positive cases. The presence of macroscopy hotbeds was visible with white colour and in the nodules with diameter of 0,1-1,0 cm in special organs or more than one organ. The hunters should be warmed for the presence of tularemia. The presence of the macroscopically visible grey colored in white hotbeds in the nodules with diameters of 0.1 -1.0 cm in the body, more or less the same as the paralyzing agent and the agent for the presence of tularemia. The results of the immunohistochemical examination are presented considerably lower compared with the results of the same alternative, but in rabbit samples that resulted seropositive. Only 12 samples (0.6%) of the examined tissues with the immunohistocation of rabbits were found positive with a lesion in the organs where they were found to be affected at least in one of the observed organs.

Keywords: Wild rabbit, meat, samples, methods, Macedonia.

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Introduction

Tularemia is an infection caused by the Gram-negative bacteria *Francisella tularensis*. The organism is named after Edward Francis who, while in Tulare County, California, helped discover how the disease was spread and how the causative organism could be cultured. Tularemia is usually a disease of animals. Humans can acquire tularemia when they come in contact with infected animals or are bitten by insects that have fed on an infected animal. Ticks, biting flies, and mosquitoes have been shown to transmit tularemia between animals and humans. Tularemia is one of several tick-borne diseases and can be spread by many species of haematophags. Tularemia, or rabbit fever, is a bacterial zoonosis caused by *Francisella tularensis*. *F. tularensis* causes serious septicemia in animals, especially wild rodents and lagomorphs (rabbits and hares), and potentially fatal, multisystemic disease in humans. The human mortality rate can reach 30% in untreated persons. *F. tularensis* is listed as a category A bioterrorism agent by the Centers for Disease Control and Prevention alongside the causative agents of anthrax, plague, smallpox, botulism, and viral hemorrhagic fevers. Generally, lesions associated with septicemic tularemia include multifocal 1–2-mm, white foci of necrosis in the liver, spleen, lymph nodes, and lungs. Tularemia is found at least 110 different kinds of animals, 25 kinds of birds and a few kinds of fish. It is found in hard-bodied ticks of the genus *Ixodes, Dermacentor, Haemophysalis* spp. It is also seen in arthropods**,** in mites and in the flies. They find shelter in the mites, and fleas and they get transported. Biovar A is met at lagomorphs, whereas holoarctic biovar B is met mostly at small rodents. Many routes of human exposure to the tularemia bacteria are known to exist. The common routes include inoculation of the skin or mucous membranes with blood or tissue while handling infected animals, the bite of an infected tick, contact with fluids from infected deer flies or ticks, or handling or eating insufficiently cooked rabbit meat. Less common means of spread are drinking contaminated water, inhaling dust from contaminated soil or handling contaminated pelts or paws of animals. Tularemia is not spread from person to person. The animals with very high sensitivity are the 1st grade reservoirs and epizootic before all, contagious for the humans and they create infective foci.

Materials and Methods

For the realization of this study were taken in 295 tissue and suspected rabbit organs and 70 samples from meats at the postmortem hares. The organs collected from rabbits served for public consumption include heart, pericardium, lung, liver, spleen, kidney, small intestine, and bone marrow, testes and epididymis, ovaries, milk glands and mediastinal lymph nodes. From formalin-fixed tissues, unparaffined microscopic stripes of tissue and organ samples were prepared. From them were prepared microscopic stripes that were stained with the classic hematoxylin eosin and ABC

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method. The immunohistochemical staining Avidin-Biotin Complex (IHC-ABC) method was applied for the demonstration of the lipopolysaccharide antigen of *F tularensis* in tissue sections. Immediately after the antigen deparafinisation and extraction (in an microwave oven in 750 W for 20 minutes in citrate tampon, pH 6,0), the lesions were incubated in solution 3% H2O2 for 10 minutes in pH 6,0 and then in a solution of 2% skimmed milk for 20 minutes. The samples were incubated during the night in 37^0 C, with a proportion 1: 6000 of the dilution of *F tularensis.* Lipopolysaccharide with the monoclonal-specific antibodies produced in rats (clones FB11 and T14,MAB8267, Chemicon International Inc, Southhampton, UK) the fusion of antibodies were detected by means of peroxidase reaction and use of specific substrate, a reaction which is exposed with dark brown colour (EnVisionb Kit, Dako, Glostrup, Denmark). A series of lesions were incubated with phosphate buffer solution and it will be used as a negative control. We used the immunohistochemical avidin-biotin complex method (IMHC-ABC) which was realized on the formalin-fixed tissues respecting the association protocol of the kit.

Results and Discussion

In this task were realized Immunohistochemical examinations of 295 tissue and suspected rabbit organs were performed and 70 rabbit meat samples sampled by hunters and restaurants serving wild goat meat in Macedonia. In addition to physical control, samples were taken and sampled in experimental rabbits and restaurants to undergo diagnostic tests for tularemia. The study carried out an assessment of suspicious organs and specimens in wild rabbit meat for this disease and the conditions of retention and consumption by hunters and other consumers who bring this product to slaughterhouses and restaurants located in the Republic of Macedonia. After obtaining results of serology and meat control killed in hunting seasons and in restaurants, we compared the data of our study with the public health data for the correlation between the pertinence of tularemia in our cases with the presence of tularemia in humans. Public health data in Macedonia are used to accomplish this task. Based on our study, we will also make recommendations for Macedonia's veterinary service, for defense strategy and disease eradication.

Tissues from Rabbits Serving for Human Consumption

Seronegative rabbit tissue samples served as a negative control for histological and immunohistochemical examination. All carcasses were evaluated under adequate biosecurity conditions at the Veterinary Laboratory. Relying on kidney fat, carcasses were rated in three categories (moderate moderate good). The tissue samples were collected to be examined fresh or stored in 10% buffered form for histological and immunohistochemical examination. The organs accumulated from rabbits served for

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public consumption include heart, pericardium, lung, liver, spleen, kidney, small intestine, and bone marrow, testes and epididymis, ovaries, milk glands and mediastinal lymph nodes. From fixed tissues to formalin, microscopic samples of tissues and organs were prepared. From them were prepared microscopic strings that were stained with the classic hematoxylin eosin and ABC method. The prepared strings were examined in a light microscope laboratory.

Table no.1. The appearance of histological lesions and lipopolisaccharide antigens *Francisella tularensis* in wild rabbits (n = 295) with a affected organ.

No	Organs	Number of positive samples	%
1	Lungs	5	2.18
2	Pericardis	2	0.68
3	Lymph nodes	2	0.68
4	Kidney	1	0.34
5	Liver	0	0
6	Milk glands	1	0.34
7	Testicles	1	0.34
8	Amount	12 (2065)	0.6

The results of the immunohistochemical examination are considerably lower compared with the results of the same alternative, but in rabbit samples that resulted seropositive. Positive with a lesion in the organs, only 12 samples (0.6%) of the tissues examined with the immunohistocation of rabbits were found to be affected at least in one of the observed organs. Positive immunohistochemistry is dominated by 5 positive cases (2.18%).

The presence of macroscopically visible fireworks in gray to white and nodules with a diameter of 0.1 to 1.0 cm in specific organs or in more than one body should warn the hunters and the diagnostic for the presence of tularemia. In these fireplaces should be considered a tularemia infestation until the time when the opposite can be proven.

The most commonly affected organs resulted in lungs, pericarditis, and kidneys, unlike other rodents and lagomorphs, where the liver and spleen result to be the main targets. In general, histologic lesions resemble the subacute tularemine reported in experimentally infected home hare.

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Table no. 2. Presentation of histological lesions and lipopolisaharid antigens *Francisella tularensis* in wild rabbits (n = 295) with two affected organs.

Nr	Organs	Number of positive samples	%
1	Lungs and pericard	2	0.68
2	Lungs and mediastinal lymph glands	1	0.34
3	Lungs and kidney	2	0.68
4	Mushkeri dhe melcia	2	0.68
5	Lungs and milk glands(testicles)	1	0.34
6	Amount	8 (1475)	0.54

With very small values compared to seropositive rabbit organs but again the lungs dominate the rat of positive sample organs in wild rabbits. Clinical evaluation of the two organs occurred between the lungs and the other observed organs. Total positive immunostimulatory specimens dominate lung and pericardium, kidney and liver with two positive cases (0.68%). This finding confirms that the species of tularemia bacterium circulating in wild rabbits in Macedonia has tropisms for the lungs.

Table no. 3. Presentation of histologic lesions and lipopolysaccharide antigens *Francisella tularensis* in seropositive wild rabbits (n = 295) with three affected organs.

No	Organs	Number of positive samples	%
1	Lungs, pericard and mediastinal lymph nodes	1	0.34
2	Lungs, pericard and testicles	1	0.34
3	Lungs, lymph nodes and testicles	1	0.34
4	Amount	3 (885)	0.34

Observing imunohistochemical observation data with simultaneous tularemia lesions, we notice that the most common cases of tularemia in the combination of lung, pericardial and mediastinal lymph glands dominated by the combination of lung, pericardial and testicular and lumbar combination, mediastinal lymph glands and testicular u are found in only one case of examination of the organs of clinically normal rabbits in Macedonia (0.34%). Observing immunohistochemical data with simultaneous lesions of tularemia in four or more rabbit organs we noticed that no positive cases were found in any case. The advantage of IHC in relation to serological tests and the bacterial culture method is that the possibility of tissue tissue infection is eliminated from the time of laboratory examination due to diving into the formalin.

So these examinations in the conditions of wild rabbit control result to be the most useful and sensitive method for the detection of *F. tularensis* in domestic animals and wild animals. The specificity of the lipopolysaccharide of *F. tularensis* used in the present study was a mixture of mouse monoclonal antibodies and virtually ensured

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the detection of all subspecies of *F. tularensis.* This was best seen in the detection of positive tissue for inflammation in clinically normal rabbit organs. The reagent did not react with other bacterial pathogens that could also cause infections in the internal organs of wild rabbits. In wild rabbits, the *F. tularensis* antigen is always associated with tissue lesions, particularly with necrotic foci. In areas of granulomatous inflammation, the bacterial antigen was present in low doses and some samples were completely absent, which may be explained by the successful elimination of bacteria from the rabbit immune system.

The main pathologic feature of the disease was the presence of fewer in number of necrotic fire in various organs and in a limited number of organs. Necrotic wounds were visible in 1 body (12 cases), 2 organs (8 cases), and in many organs (4 cases). The fires were scattered irregularly, well marked, round, white, slightly gray in white, or yellow in white, with a diameter of 0.1 to 0.5 cm, and they were often surrounded by one dark hyperemial ring. in some cases. Lungs in the lungs and kidneys were joined to nodules with a diameter of up to 1 cm. The foci are often observed on the surface of the lung serosis and in the kidneys. These fireplaces and hearths were raised in dome or flat shape, moreover, the surface of the serosis was dry and crushed, and so these wounds resemble serous spores. The affected testicles were enlarged, and some yellow-colored bricks in the white or nodules were visible on their cut surface. The presence of fireplaces in clinically healthy rabbit samples was not observed in the bone marrow and in the lactation gland. Some of the cases that resulted positive from these samples were adult male rabbits with poor body condition. They had lungs, kidneys, melts, and spleen enlarged. in the lungs were observed petochemical hemorrhage, and a large number of gray-green foci in white or yellow in white were observed in both lungs and pericarditis, as well as on testicles in one case. Seronegative rabbits are generally not observed lesions similar to tularemine or other infections. The fountains identified by the general pathological examination respond to granulomatous inflammation, which completely replaced the normal tissue structure of the affected organs. These foci are scattered irregularly in the organs and serosis of the serous membranes was often involved. Macrophage cells were the dominant type in the composition, but other cells were occasionally found, including lymphocytes, heterophile granulocytes, giant multigrain cells, and fibrocytes. Vaginal or multifactorial necroses have been observed frequently and were at the center of these lesions. Granulomatous inflammation was found by microscopic examination but not by general pathological examination in the mediastinal lymph nodes. In some cases, the granulomatous inflammation in the lung did not contain any or only small necrotic necrosis. Vascular remains and bronchioles necrotic are observed in the affected areas. The secretions and inflammatory remains are found in some bronchioles not adjacent to areas of granulomatous inflammation. Alveolar edema was present in these areas. The heartbone of the heart showed pronounced

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central necrosis, and fibrin inflammation was evident on serous surfaces. Extensive granulomatous inflammation was encountered in the cortex and in the affected kidney. The female tubulas found in these areas were partly or completely necrotic and filled with necrotic remains. Multifocal granulomatous inflammation was found in the subcortical area of the kidney basin and necrotic inflammatory exudates were present in the lumen. In testicles and epididymitis, granulomatous inflammation has expanded the intertubular binding of the connective tissue over large surfaces.

Some tubes were partially or completely necrotic, and their lumen was filled with inflammatory necrotic exudates. Focal granulomatous inflammation with central necrosis have been found in the liver, bone marrow, milk glands, spleen, and mediastinal lymph nodes (Table 2). Histological lesions were essentially the same with cases of natural mortality and hunted animals. However, in comparison with some of the cases observed in seropositive rabbits, numerous necrotic foci were evenly distributed in 2 to 3 maximum organs. Results of the immunohistochemical method Avidin-Biotin Complex (IHC-ABC) were successfully used for microscopic control of samples obtained from hunters, users and traders of wild hare in Macedonia during 2015-2016. By immunohistochemical examination, *F. tularensis* antigen was detected in all cases with tissue with lesions (4 out of 70 samples 5.7%) (Table). During our research 70 samples of rabbit tissue killed by Macedonian hunters in Debresh, Nerove, Allbance, Presille, Bellushine, Haracine, Tearce, etc. were examined. These samples were collected from shops and restaurants serving wild rabbit meat in their menu. The number of samples collected was considerable considering that the population of the above areas does not generally prefer the consumption of wild rabbit meat. The rabbit is consumed by many hunters and their family members and by some luxurious restaurants. The wild hare in Macedonia is also used in tourist areas. Samples collected were sent to the SKE Laboratory at the Faculty of Veterinary Medicine, in which 70 samples were formally fixed according to protocol accompanying the kit.

Table no.4. Evaluation results of wild rabbit carcasses used for consumption in Macedonia.

Nr	Condition	Number of cases	% to total
1	Very good and good	38	54.3
2	Medium	25	35.7
3	Weak	7	10
4	Amount	70	100

The body condition of rabbits was good and very good in 38 rabbits (38 out of 70 cases, 54.3%), averages 25 rabbits (25 out of 70 cases, 35.7%) and weaker in only 7

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rabbits (7 out of 70 cases, 10%). The carcass observation results indicate a good physical condition of the wild hare population in Macedonia.

Table No. 5. Results obtained from the immunohistochemical examination of wild rabbits destined for public consumption in Macedonia in 2015-2016.

No.	The source of tissue acquisition	Sampling period	Nr. of the samples	Positive sample	Negative sample
1	Huntress	2015	21	-	21
2	Huntress	2016	24	3	21
3	Restaurants	2015	17	1	16
4	Restaurants	2016	8	-	8
Amount			70	4 (5.7%)	66

Bacterial antigen of *F. tularensis* was found only in 4 samples (5.7%). Three samples were champion rabbits hunted in 2016. A positive mucus *F. tularensis* was found only in a restaurant in 2015. No *F. tularensis* case was detected in rabbits consumed by hunters in 2015. Also no case was found positive for *F. tularensis* antigen in restaurants during 2016. So the four animals sampled by hunters and restaurants that resulted in immunohistochemical examination are a small number and adding this and the low pathogenicity in circulating yeast seems that rabbit meat used by hunters and restaurants in Macedonia is not a risk for public health. Compared with the examinations of the slimming agglutination test, the immunohistochemical examination shows 100% sensitivity, but only 83% specificity. Predictable negative test value was 100%, while predictive value of positive testing was 92%. The bacterial antigen apparently looked like a thin intracellular granulocyte structure within intact alveolar epithelial cells, hepatocytes and intravascular macrophages, while extracellular stain was present in the blood vessels. Seronegative animals and negative control tissue cuts generally showed the results to remain negative for wild rabbit samples used for public consumption. One of the reasons is the adaptation of the bacterial species that circulates in Macedonia and the finding of a symbiosis between him and the rabbit immunity.

Tularemia and Public Health Risk in Macedonia

From the Central Institute of Public Health in Macedonia, we received data on the circulation of tularism in years. The results show that tularemia in humans has always been present with variations but generally mild as a clinical presentation. There are no special years and the main characteristic of its emergence in certain areas touching significant groups of people. Generally, people affected by tularemia in Macedonia are people found in rural areas. According to the profession, the first are hunters and

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people who work with coffins. There is no high figure of people affected in restaurants, because generally and this varies according to wild meat rabbit areas does not prevail much in Macedonia. Special areas and tourist resorts are characteristic for the Macedonian wild goat meat menu. Contagious contact with infected sticks is always an option of infecting people in rural areas. According to the database, the first cases of tularemia registered in Macedonia belong to the 1990s. In 1990, 5 cases were recorded in people in the Municipality of Berovo. These were cases distributed and recorded in various fireplaces in the villages of Mitrashince and Robove. In the village of Budinarce 3 cases were recorded this year. In 1995 an epidemic was recorded in the village of Mitrashinice, Belishove municipality. In this epidemic 31 individuals were registered as ill. In 2010 there was an epidemic of several confirmed and unconfirmed cases of tularyme involving many villages in Gostivar. Suffice it to recall that only in the village of Vrapciste during this epidemic were confirmed to affect 20 people. Almost every year there are cases of an echo that varies greatly between the years, but with a minimum of almost constant cases. In 2011, as one of the years with the minimum of positive cases, only one positive case was registered in Skopje. Data for Tularemia infection in Macedonia for 2017 show that it was affected by a total of 13 cases -10 from the public health center in Skopje and 3 cases reported by the Peoples Hospital in Kumanovo. The tularemia epidemic during 2017 has not been declared. According to sex during 2017, tularemia in humans affects 6 males and 7 females.

Table no. 6. Results of infected people from tularemia in Macedonia for 2017 by age group.

No	Age group	Number of cases with tularemia
1	1-4	1
2	5-9 vjec	1
3	10-14	2
4	20-29	2
5	30-39	3
6	40-49	1
7	50-59	2
8	60>	1
9	Amount	13

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Figure no. 1. Data on the presence of tularemia in people in Macedonia.

Conclusions

The results of the immunohistochemical examination are considerably lower compared with the results of the same alternative, but in rabbit samples that resulted seropositive. Only 12 samples (0.6%) of the examined tissues with the immunohistocation of rabbits were found positive with a lesion in the bodies where they were found to be affected at least in one of the observed organs. Positive immunohistochemistry is dominated by 5 positive cases (2.18%). Clinical evaluation of the two organs occurred between the lungs and the other observed organs. Total positive immunostimulatory specimens dominate lung and pericardium, kidney and liver with two positive cases (0.68%). Results of the immunohistochemical observation with simultaneous lesions found the combination of lung, pericardial and mediastinal lymph glands with lung, pericardial and lung and lung, mediastinal lymph glands and testicles in only 1 case of clinically normal samples (0.34%). No cases were found in immunohistochemical observation with simultaneous lesions of tularemia in four or more bodies of wild rabbits. The carcass observation results indicate a good physical condition of the wild hare population in Macedonia. The body condition of rabbits was good and very good in 38 rabbits (38 out of 70 cases, 54.3%), averages 25 rabbits (25 out of 70 cases, 35.7%) and weaker in only 7 rabbits (7 out of 70 cases, 10%). Bacterial antigens and *F tularensis* was found only in 4 samples (5.7%) sampled by wild rabbits used for consumption by hunters and restaurants in Macedonia. Three samples were rabbits sampled by hunters and fired in 2016. A positive *F. tularensis*

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was found only in a restaurant in 2015. No *F. tularensis* case was discovered in rabbits consumed by hunters in 2015. Also no positive case was found for *F. tularensis* antigen in restaurants during 2016. In Macedonia there have been considerable fluctuations cases of tularemia in one. Full post-1990 recordings show that tularemia in humans is encountered in people living and working in rural areas that come into contact with infected cofetins and ticks. Meanwhile, rabies-infestation cases are not problematic as a consequence of their lack of preference and its generally low consumption in Macedonia. Samples of rabbits from hunters and restaurants that resulted in positive immunodeistochemical examination resulted in a small number. Adding this and the low pathogenicity in circulating shrimp appears that rabbit meat used by hunters and restaurants in Macedonia does not pose a high risk to public health.

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