IDENTIFICAÇÃO DE FOCOS DE TULAREMIA USANDO IMUNOENSAIO ENZIMÁTICO NAS PLUMADAS DE AVES PREDADORAS

DETECTION OF FOCI OF TULAREMIA USING ENZYME IMMUNOASSAY FOR THE PREDATORY BIRD PELLETS

ВЫЯВЛЕНИЕ ОЧАГОВ ТУЛЯРЕМИИ С ИСПОЛЬЗОВАНИЕМ иММУНОФЕРМЕНТНОГО АНАЛИЗА ПОГАДОК ХИЩНЫХ ПТИЦ

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RESUMO

Os resultados da identificação de focos de tularemia usando as plumadas de aves predatórias na parte central da Rússia usando o exemplo da República da Moldávia são apresentados. A eficácia da detecção de tularemia nas plumadas em comparação com outros biomateriais. O conteúdo médio do antígeno da microbiota tularemia das plumadas de aves de rapina foi de 9,4%. Os resultados do imunoensaio enzimático das plumadas de diferentes espécies de aves de rapina para a presença do agente causador de tularemia mostraram que em algumas espécies há mais casos de detecção. O maior número de registros do agente causador de tularemia nas plumadas foi observado em corujas de águia (29,2%). Em menor grau, o patógeno foi detectado na coruja de cauda longa (14,7%). O antígeno da tularemia foi encontrado mais frequentemente nas plumadas de aves de rapina do que no biomaterial de roedores. A maior proporção de amostras positivas foi registrada em áreas costeiras. Na dinâmica de identificação do agente causador de tularemia deve-se notar em 2015, 2017 e 2018, que são caracterizadas por altas taxas.

Palavras-chave: *Tularemia, Cirurgia, Epizootiologia, Imunoensaio*

ABSTRACT

The results of identifying tularemia foci using the predatory bird pellets in the central part of Russia using the example of the Republic of Mordovia are presented. The efficacy of tularemia detection in pellets compared with other biomaterials has been shown. The average content of the tularemia microbe antigen from the predatory bird pellets was 9,4%. The greatest number of registrations of the causative agent of tularemia in the pellets was observed in Eagle Owl (29,2%). To a lesser extent, the pathogen was detected in the Ural Owl (14,7%). The tularemia pathogen was detected more often in the bird predator pellets than in the biomaterial from rodents. The largest share of positive samples was recorded in the riverine districts. The dynamics of identifying the causative agent of tularemia should be noted in 2015, 2017 and 2018, which are characterized by high values of the indicator.

Keywords: *Tularemia, Pellet, Epizootology, Enzyme immunoassay*

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АННОТАЦИЯ

Представлены результаты выявления очагов туляремии с использованием погадок хищных птиц в центральной части России на примере Республики Мордовия. Показана эффективность выявления туляремии в погадках по сравнению с другими биоматериалами. Среднее содержание антигена туляремийного микроба из погадок хищных птиц по Мордовии составило 9,4 %. Результаты иммуноферментного анализа погадок разных видов хищных птиц на наличие возбудителя туляремии показали, что у некоторых видов больше случаев выявления. Наибольшее число регистрации возбудителя туляремии в погадках отмечено у филина (29,2%). В меньшей степени возбудитель выявлялся у длиннохвостой неясыти (14,7%). Туларемийный антиген обнаруживался чаще в погадках хищных птиц, чем в биоматериале от грызунов. Наибольшая доля положительных образцов была зарегистрирована в прибрежных районах. В динамике выявления возбудителя туляремии следует отметить в 2015, 2017 и 2018 года, которые характеризуются высокими показателями показателя.

Ключевые слова: *Туляремия, Погадка, Эпизоотология, Иммуноферментный анализ*

INTRODUCTION

Tularemia is a zoonosis affecting more than 120 species of vertebrate animals, mainly representatives of the order of rodents. It is a natural focal disease widespread in Europe (Cerny, 2001; Tärnvik *et al.*, 2004; Aldea-Mansilla et al., 2010). In various regions of Russia, the value of tularemia is also great (Trankvilevskii *et al.*, 2015; Kudryavtseva *et al.,* 2016). Tularemia is an infection relevant to the Russian Federation as before. The changes in the epizootic and epidemic process of tularemia necessitate better surveillance as a whole and epizootiological monitoring in particular (Nafeev *et al.,* 2016). Pathogenicity of tularemia (*Francisella tularensis*) belongs to group II of microorganisms, the isolation and identification of which can be carried out in specially equipped laboratories by trained specialists vaccinated against tularemia. Tularemia is transmitted to a person by contact (when rodents are opened, when in contact with contaminated water), alimentary (when consumed, food and water contaminated by rodents) and aspiration (by dust inhalation) (Magnino *et al.,* 2011). The pathogen can also be transmitted by blood-sucking and other arthropods (ticks, fleas, mosquitoes, gadflies, and flies). For to identify the causative agent of tularemia in the environment, for monitoring purposes, rodents and their nests, water from natural sources, and birds of prey are analyzed. In the latter case, experts, as a rule, do not attach any importance to what type the casting belongs. The potential role of birds of prey is can as asymptomatic carriers of pathogenic bacteria which could be disseminated in the environment not only through the birds of prey feces but also through their pellets (Dipineto *et al.,* 2015).

Serological analysis (reaction of antibody neutralization) of pellets of birds for the presence of tularemia microbe antigen is an efficient method of detection and investigation of epizootics in all types of natural foci of the infection and also for the exploration of new focal territories. The method permits with small expenditure of labour and within a short time, to collect material characterizing the epizootic process on a large territory (Dobrokhotov and Meshcheryakova, 1980). Proved that the greater effectiveness of examination of bird's pellets for the reconnaisance investigation of tularemia in comparison with the bacteriological methods applied usually.

We have proposed a hypothesis according to which pellets of certain species of birds of prey give different results on the identification of the causative agent of tularemia. The reason for this is the differences in the food spectrum of different species of predators. In the literature available to us, we have not found studies relating specifically to this problem. In one of the regions of the European part of Russia, namely in Mordovia, an earlier analysis was

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not conducted to identify the causative agent of tularemia in the biomaterial from specific species of birds of prey.

The aim of our research was to identify the causative agent of tularemia in the pellets of owls and day birds of prey in the Republic of Mordovia. The results of the analysis of the collected material provide new information describing the possibility of using tularemia for monitoring certain species the bird predator.

MATERIALS AND METHODS

The material for this article was the Eagle Owl *Bubo bubo* (Linnaeus, 1758), the Ural Owl *Strix uralensis* (Pallas, 1771), the Long-eared Owl *Asio otus* (Linnaeus, 1758), the Imperial Eagle *Aquila heliaca* (Savigny, 1809), the White-tailed Eagle *Haliaeetus albicilla* (Linnaeus, 1758), collected in the snowless period of 2015-2018 at the breeding sites in Bol'shebereznikovskii (54°04' N, 46°42' E), Dubenskii (54°20' N, 46°32' E), Kochkurovskii (53°53' N, 45°22' E), Chamzinskii (54°25' N, 45°34' E), Atyashevskii (54°39' N, 46°19' E), Ardatovskii (54°47' N, 46°08' E) districts of the Republic of Mordovia. In the Bol'shebereznikii district, pellets were collected in biotopes in the vicinity of the villages of Simkino, Tazino, Veise, Nerley, Shugurovo. In the Dubenskii district, the collection of pellets was carried out in the biotopes of the surroundings of the villages of Nikolaevka, Kaibichevo, Cheberchino, Morga, Krasino, Krasnoe Pol'tso. In Kochkurovskii district, a pellet collection was carried out in biotopes of the surroundings of the villages of Novyi Turdaki, Bulgakovo. In the Chamzinskii district, the collection of pellets was carried out in biotopes of the vicinity of the villages of Makolovo and Machkazerovo. In Atyashevskii district, the collection of pellets was carried out in biotopes of the surroundings of the villages of Dyurki. In Ardatovskii district, the collection of pellets was carried out in biotopes of the vicinities of the villages of Redkodub'e, Lun'ga. The material on nutrition was collected both purposefully and in the course of field work on the study of the spectrum of nutrition. Samples were collected and transported in plastic bags. Pieces were subjected to enzyme immunoassay (EIA) to identify the causative agent of tularemia. Samples were analyzed at the accredited testing laboratory center of the Center for Hygiene and Epidemiology of the Republic of Mordovia. The method is based on the scheme of indirect enzyme immunoassay. The use of an enzyme immunoassay for the diagnosis of tularemia and for the detection of the pathogen in environmental objects is regulated by guidelines (Balahonov *et al.*, 2011). In the formulation of enzyme immunoassay, the tularemia antigen contained in the test material specifically interacts with tularemia immunoglobulins sorbed on the plate. The resulting antigen-antibody complex was detected using a conjugate that catalyzed the splitting of ortho-phenylenediamine. Preparation for the study of the pellets of birds of prey was carried out as follows. Each bead was individually poured with 1% formalin so that its excess was 5-10 ml. Then the knead kneaded. After settling for 1-6 hours, the supernatant was collected, heated at a temperature of 100 ° C for 20 minutes and, if necessary, filtered through a cotton swab.

EIA was performed in the wells of a sensitized plate. For this purpose, 0.1 ml of tularemia immunoglobulins of the working dilution were added to the wells of an EIA analysis using an automatic dosing unit. The plate with immunoglobulins was closed with a lid and incubated at 37 ºC for 3 hours or at 5ºC for 16-18 hours. At the end of the incubation, the contents of the wells were removed by shaking into a container for used solutions. 300 microlitre of phosphate-saline buffer and tween-20 were added to each well. Then mixed with five to six circular motions of the tablet on the surface of the table. After that, the contents of the wells were removed by decanting. Carefully remove the remnants of moisture from the wells, several times abruptly shaking out the plate on a gauze napkin or filter paper.

For direct analysis, 0,1 ml of a

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suspension of a tularemia microbe from the kit (positive control) was added to the two wells with a dispenser. 0,1 ml of phosphate-saline buffer with tween-20, bovine serum albumin was added to two wells and used as a negative control. In the remaining wells, 0,1 ml of the tested suspensions were introduced at a concentration of 1,0 × 109 m.s. / ml or the material under investigation in two replications.

The tablet with the suspension of cultures and the test material was closed with a lid and incubated for 60 min at 37 ° C.

At the end of the incubation, the contents of the wells were removed by simply shaking into a container for used solutions. 300 microlitre of phosphate-saline buffer with tween-20 was added to each well. Stirred with five to six circular motions of the plate on the surface of the table, after which the contents of the wells were removed by decanting. The operation was repeated twice. After that, the remnants of moisture were carefully removed from the wells, several times sharply shook out the tablet on a gauze napkin or filter paper.

0,1 ml of the dilution of the working dilution was added to all wells. The tablet with the introduced conjugate was closed with a lid and incubated for 30 min at 37 ºC.

Removed solutions from the wells of the tablet by simply shake out in the tank for used solutions. Washed in phosphate-buffered saline with tween-20 six times.

In all the wells in the same sequence as the conjugate, was made of 0.1 ml of the substrate-indicator solution. We took into account the color change of the solutions in the wells for 1-3 minutes, using a stopwatch. In the negative control wells, the liquid should remain colorless or slightly yellow. In wells with dilutions of suspensions and the test material, the liquid takes on an orange to yellow color, depending on the concentration of microbial cells in the sample.

As soon as the liquid in the negative control wells started to turn slightly yellow, the reaction was stopped by adding 50,0 microlitre of stop solution to each well of the plate at the same rate and in the same sequence as the substrate-indicator solution, and the contents of the wells were thoroughly mixed.

The results were recorded on a photometer for enzyme immunoassay at a wavelength of 492 nm. In this case, the duration of the procedure for measuring the optical density should not exceed 15 minutes. The results were considered positive if the optical density of the samples was 2 or more times higher than the optical density of the negative control.

A total of 341 pellets from nesting parts of the Eagle Owl (n = 142), the Ural Owl (n = 79), the Long-eared Owl (n = 59), the Imperial Eagle (n = 37), the White-tailed Eagle (n = 24) were subjected to analysis. The big share of pellets of an Eagle Owl is assembled caused by close attention to studying of the different parties of biology of a look in the region (Andreychev et al., 2017; Lapshin et al., 2018). Statistical calculations were performed using computer programs AtteStat 8 (2002), Microsoft Office Excel (2003).

RESULTS AND DISCUSSION:

The average content of the tularemia microbe antigen from the pellets of birds of prey in Mordovia was 9.4%. The results of the enzyme immunoassay of the pellets of different species of birds of prey for the presence of the causative agent of tularemia showed that in some species there are more cases of detection. The greatest number of registrations of the causative agent of tularemia in the pellets was observed of the Eagle Owl (29,2%). To a lesser extent, the pathogen was detected of the Ural Owl (14,7%). The tularemia pathogen has not been identified in the graves of the Long-eared Owl, the Imperial Eagle, the White-tailed Eagle.

The Eagle Owl and Ural Owl can be recognized as monitoring bird species for the detection of tularemia in natural focis. The reason for this is a wide range of prey and especially small mammals. According to the results of the work in the pellets and eaters of the Eagle Owl, mammals prevailed (88,5%) over birds (8,1%) (Andreychev *et al.,* 2014;

2016). Mammals in the spectrum of Eagle Owl in Mordovia are represented by the following: the common vole *Microtus arvalis* (Pallas, 1778), the short tailed vole *M. agrestis* (Linnaeus, 1761), the root vole *M. oeconomus* (Pallas, 1776), the bank vole *Clethrionomys glareolus* (Schreber, 1780), the water vole *Arvicola terrestris* (Linnaeus, 1758), the muskrat *Ondatra zibethicus* (Linnaeus, 1766), the common hamster *Cricetus cricetus* (Linnaeus, 1758), the Norway rat *Rattus norvegicus* (Berkenthout, 1769), the Ural field mouse *Sylvaemus uralensis* (Pallas, 1811), the yellow-necked mouse (*S. flavicollis* (Melchior, 1834), the striped field mouse *Apodemus agrarius* (Pallas, 1771), the harvest mouse *Micromys minutus* (Pallas, 1771), the northern birch mouse (*Sicista betulina* (Pallas, 1778), the great jerboa *Allactaga major* (Kerr, 1792), the spotted suslik *Spermophilus suslicus* (Gueldenstaedt, 1770), the red squirrel *Sciurus vulgaris* (Linnaeus, 1758), the hedgehog *Erinaceus* sp. (Linnaeus, 1758), the mole *Talpa europaea* (Linnaeus, 1758), the Eurasian common shrew *Sorex araneus* (Linnaeus, 1758), the ostsibirische shrew *S. isodon* (Turov, 1924), the water shrew *Neomys fodiens* (Pennant, 1771), the weasel *Mustela nivalis* (Linnaeus, 1766). According to the results of work in the pellets and eating the Ural Owl, mammals (86,4%) prevailed over birds (13,6%) (Andreychev and Lapshin, 2017). Mammals in the food spectrum of the Ural Owl in Mordovia are represented exclusively by members of the Rodentia order. The following species of rodents were identified: the bank vole (*Cl. glareolus*), the common vole (*M. arvalis* s.l.), the root vole (*M. oeconomus*), the Ural field mouse (*S. uralensis*), yellow-necked mouse (*S. flavicollis*), and the striped field mouse (*A. agrarius*). Most of the diet of the Ural Owl falls on gray voles (47,7%), namely the common vole (41,8%) and the vole-housekeeper (5,9%). There is a similarity of nutrition with the Eagle Owl (Andreichev *et al.,* 2014) and the Imperial Eagle. The bank vole stands in second place in the diet of the Ural Owl (31,4%). The share of mice accounted for only 7,3%. Of mice, the predator most often harvests the Ural field mouse. The insignificant predominance of open spaces over forest representatives among the victims is explained by the fact that the Ural Owl meadow for hunting prefers, first of all, glades, forest edges and edges of cuttings or burns adjacent to the forest. Thus, it can be stated that the range of feeding of the Ural Owl can vary by year depending on the number of gray and red vole species. However, it is possible to precisely name the common and the bank vole as the main prey of *S. uralensis* in Mordovia. Our data are consistent with the results of other researchers (Obuch *et al.,* 2013).

Birds (54%) prevailed over mammals (46%) in food spectrum the Imperial Eagle. Mammals in the feeding spectrum of the Imperial Eagle in Mordovia are represented by the following: the common vole (*M. arvalis* s.l.), the water vole (*A. terrestris*), the muskrat (*O. zibethicus*), the common hamster (*Cr. cricetus*), the Norway rat (*R. norvegicus*), the striped field mouse (*A. agrarius*) the hedgehog (*Erinaceus* sp.), the cat *Felis catus* (Linnaeus, 1758). In the feeding of the White-tailed Eagle, birds (26%) prevailed over mammals (less than 15%). Mammals in the food spectrum of the white-tailed eagle in Mordovia are represented by the following water vole (*A. terrestris*), the muskrat (*O. zibethicus*), the Eurasian beaver (*Castor fiber*), the common red fox (*Vulpes vulpes*). The following species have been identified in the food of an eared owl: the common vole (*M. arvalis* s.l.), the striped field mouse (*A. agrarius*), the Ural field mouse (*S. uralensis*).

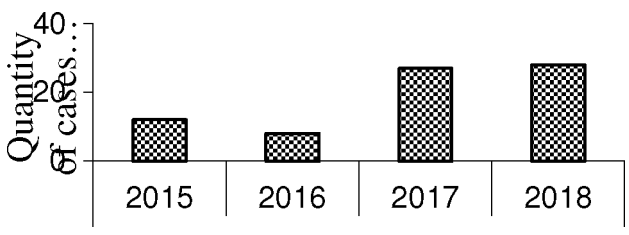

Figure 1. Dynamics of detection of tularemia microbe antigen among small mammals in the region

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Table: Efficiency of detection of the causative agent of tularemia in different biomaterials from animals

District	Detection of tularemia pathogen	
	Pellets of prey birds	Rodents
Bol'sheberezhnikovskii	+	+
Dubenskii	+	–
Kochkurovskii	–	–
Chamzinskii	+	+
Atyashevskii	–	–
Ardatovskii	+	–

The dynamics of identifying the causative agent of tularemia in Mordovia (Fig. 1) should be noted in 2015, 2017 and 2018, which are characterized by peak values. It is advisable to indicate the distribution of cases of registration of positive samples in relation to the identification of the causative agent of tularemia in the pellets of prey birds of the districts. A large proportion (66,7%) was recorded for the Dubenskii district, for the Ardatovskii district (43,8%), for the Bol'sheberezhnikovskii district (25,4%). Fewer cases of detection in Chamzinskii (15,1%), Kochkurovskii (13,8%), Atyashevskii (10,3%) districts. Mostly, the tularemia microbe antigen was most often detected in areas along the middle rivers (Sura, Alatyr) than in areas that do not have such water arteries. In pellets of prey birds, the causative agent of tularemia was detected in the districts more often than in the biomaterial from rodents (Table). This circumstance can be explained from the position that the remains of the victim can contain several victims at once. This is evidenced by the results of studies of osteological material (Andreychev *et al.,* 2014; 2016; Andreychev and Lapshin, 2017). Therefore, the probability of detecting a tularemia microbe antigen is higher here.

Analyzing the results obtained in Mordovia (Andreychev et al., 2016) from the spectrum of mouse-like rodents infected with tularemia (Fig. 2), the primary role of the carrier of this disease of the bank vole (42%) and the Ural field mouse (25%) should be noted. The house mouse (15%) and the common vole (13%) have a slightly smaller share of tularemia infection among rodents of the Mordovia.

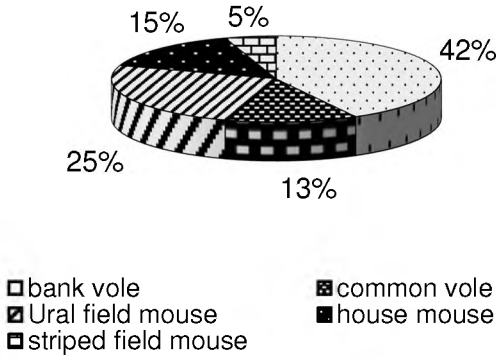

Figure 2. The structure of the population of rodents involved in the circulation of tularemia

For the Republic of Mordovia, the average content of the tularemia microbe antigen (9,4%) from the predatory bird pellets is comparable with the results of research on the Brest region (10,5%) and is significantly higher than in the Gomel region (0,8%) in Belarus (Tsvirko *et al.,* 2016).

Our results on the significant diversity of the spectrum of the Eagle Owl and the Ural Owl are consistent with the literature data. It is this circumstance that determines their significant role in the use of tularemia as monitoring. Many researchers revealed that the major part of prey in the diet of the eagle-owl consists of mammals (Gromov and Egorov, 1953; Shehab, 2004; Hofmann et al., 2005; Bayle and Prior, 2006). However, the rare bird's favorite mammalian food is rodents, as an analysis of the diet has shown (Andreychev et al., 2014, 2016). The range of Ural Owl food includes more than twenty species of mammals, thirty species of birds and a number of animals of other classes (Sidorovich *et. al.,* 2003; Kloubec *et al.,* 2005; Dravecky and Obuch, 2009). The owl's diet, like in the Eagle-owl's one, can vary quite significantly in different regions. The basis of

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its diet (20.0–85.8 %) is made up of various mammals (Sharikov *et al.,* 2009). Among the owls of the *Strix* genus the Ural Owl is able to hunt the largest animals (capercaillie, black grouse, white partridge, hazel grouse, squirrel). But its main food is the muroid rodents (Mikkola, 1983). At one pellet, we sometimes record 4 voles at once.

The results of the study of tularemia-infected mouse-like rodents in the Republic of Mordovia are consistent with data obtained throughout the Volga Federal District (Trankvilevskii *et al.,* 2015) Currently, the vole is a major carrier of tularemia in Mordovia (42% of cases infected among all rodents). It should be noted a similar picture in the primary role of the carrier of this disease of the bank vole in the Volga Federal District – 56,2%. The share of the Ural field mouse in the Volga Federal District accounts for 21,9%. The striped field mouse in the Volga Federal District accounts for 15,6%. For comparison, the main carrier of tularemia in the Smolensk region is the common vole – 46 (39%) cultures; the secondary ones are the striped field mouse – 16 (13,55%) and the house mouse *Mus musculus* (Linnaeus, 1758) – 18 cultures (15,25%) (Popov *et al.,* 2017). In the Belarusian Polesie from 2001 to 2015 the antigen of the pathogen was diluted 1: 20–1: 160 in the striped field mouse, the forest mouse, the yellow-necked mouse, the harvest mouse, house mouse, the bank vole, the common vole, root vole, water vole, the Norway rat. The leading place in the number of positive answers is occupied by: the house mouse – 147 (42,6%), the striped field mouse – 69 (20,0%), the common vole – 45 (13,0%), the bank vole – 32 (9,2%) (Tsvirko *et. al.,* 2016).

On the districts the number of positive reactions in pellets to a tularemia differed. The largest share of positive samples was registered in the riverside districts: Dubenskii (66,7% of all samples of the district) and Ardatovskii (43,8% of all samples of the district), Bol'shebereznikovskii (25,1% of all samples of the district). A similar situation of uneven detection of tularemia in the districts was also observed for Belarusian Polesye. The pathogen antigen was found regularly in 1978–2000 in the castles of birds of prey and in the litter of predatory mammals. So, in 1982, in the study of 326 specimens of the bird's food and 475 specimens of predator feces collected in 4 districts, the antigen was not detected. In 1983, the number of positive probes was 2,4%; litter 1,8%. Moreover, in two of the five surveyed areas, the antigen was not detected at all, in two the number of positively responding bugs exceeded 5,0%. In 1984, the antigen was found only in the bird pellets in 5 of the 11 areas surveyed. The number of positive samples was 5,1%. In some cases, a high percentage of positively reacted samples were noted in areas where tularemia was not recorded in the past. In areas characterized by the presence of active foci in the past, the antigen was rarely found in the pellets and excrement (Tsvirko *et. al.,* 2015).

The number of positive reactions of the Belarusian Polesye differed by year, as well as of the Republic of Mordovia. For the Belarusian Polesie in 2015, there were more cases of registration of positive reactions in the pellets, compared with 2016 and 2017. For Mordovia (Fig. 1), the number of registrations of the tularemia pathogen was also observed in 2015 compared to 2016. However, in 2017 and 2018, the number of cases of registration increased dramatically.

4. CONCLUSIONS

The average content of the tularemia microbe antigen from the predatory bird pellets was 9,4%. The greatest number of registrations of the causative agent of tularemia in the pellets was observed in Eagle Owl (29,2%). To a lesser extent, the pathogen was detected in the Ural Owl (14,7%). The tularemia pathogen was detected more often in the bird predator pellets than in the biomaterial from rodents. The largest share of positive samples was recorded in the riverine districts. The dynamics of identifying the causative agent of tularemia should be noted in 2015, 2017 and 2018, which are characterized by high values of the indicator.

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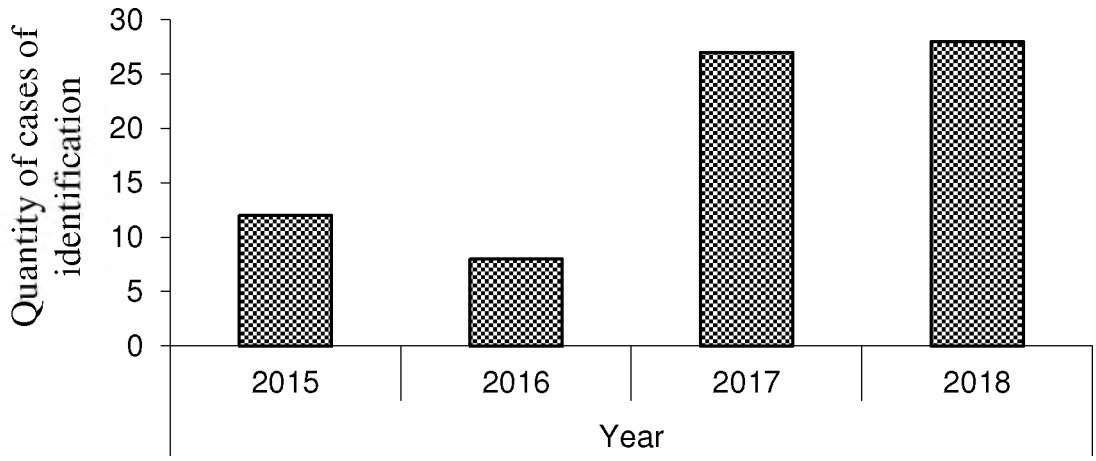

Figure 1. Dynamics of detection of tularemia microbe antigen among small mammals in the region

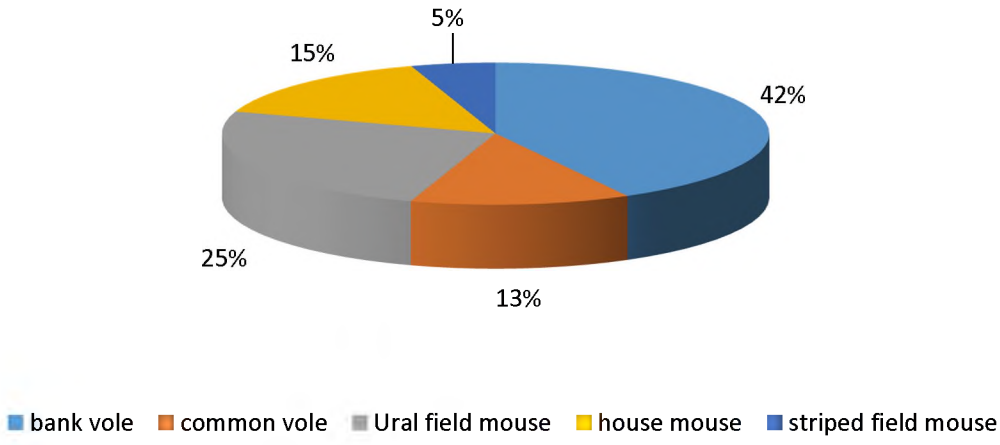

Figure 2. The structure of the population of rodents involved in the circulation of tularemia

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