VILNIUS UNIVERSITY
STATE RESEARSH INSTITUTE
CENTER FOR INNOVATIVE MEDICINE

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EPIDEMIOLOGY, DIAGNOSTICS AND IMMUNOPROPHYLAXIS OF RABIES IN WILD AND DOMESTIC ANIMALS IN LITHUANIA

Summary of doctoral dissertation
Biomedical sciences, biology (01B);
Immunology, serology, transplantation (B 500)

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This study was carried out in 2008-2012 at the Institute of Immunology of Vilnius University and, after reorganization, at the State Research Institute Centre for Innovative Medicine and at the National Food and Veterinary Risk Assessment Institute.

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The dissertation is available at the Library of State Research Institute Center for Innovative Medicine and Vilnius University Library.

VILNIAUS UNIVERSITETAS
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INOVATYVIOS MEDICINOS CENTRAS

INGRIDA JACEVIČIENĖ

LAUKINIŲ IR NAMINIŲ GYVŪNŲ PASIUTLIGĖS EPIDEMIOLOGIJA, DIAGNOSTIKA IR IMUNOPROFILAKTIKA LIETUVOJE

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Introduction

Rabies is a virus disease. It is one of the oldest and most dangerous human and animal diseases. This is a disease that is transmitted directly from animal to animal and from an animal to a human being. Human rabies is the most fatal of all ever heard-of diseases (Rupprecht, 2004). The disease is cause by a neurothropic virus belonging to the *Rhabdoviridae* family of the *Lyssavirus* genus. This is a virus containing RNR, which is mainly found in the brains of people, domestic and wild animals that are infected with rabies, it is also found in abundance in the spinal cord, salivary glands, and saliva. This disease is contracted after a rabid animal has bitten a human being or another animal or slavered the damaged skin. People usually contract this disease from stray domestic animals therefore a realistic threat is posed to everyone to become infected with the rabies virus (RV). Despite the existing preventative measures against rabies people should avoid contact with unknown or strangely behaving animals and regard the contact they have had with them as a danger to life. The early symptoms of rabies in people are non-specific: fever, shiver, a headache, general indisposition (OIE, 2008; Tortora et al., 2010). When the disease starts to progress neurological symptoms appear: anxiety, fear, insomnia, bewilderment, paralysis, hallucinations, sialorrhea, difficulty in swallowing (dysphagia), and aggression. Death usually occurs following some days after appearance of the symptoms (Dietzschold et al., 1987; Fooks et al., 2009).

The first test for rabies were carried out in the 19th century. Since then measures to protect people against this disease were taken, animal rabies control was started and epidemiology, pathogenesis of this diseases and response to immunisation were studied. In many economically developed countries the system of diagnostics of rabies is very well organised and it operates rapidly and efficiently. Very effective measures to protect people are created and systems of control and liquidation of this disease are technologically developed. However, irrespective of this, rabies still causes very significant economic losses and human deaths are quite often in the regions of Africa, Asia and South America (Bourhy et al., 1992, Schneider et al., 1994).

Predatory mammals (foxes, raccoon dogs, bats, wolves, etc.) are the main source of the rabies infection in wild fauna. Red foxes *(Vulpes vulpes),* which are one of the main spreaders of rabies virus account for the largest number (85 percent) of the positive rabies cases (Aubert et al., 1995). The second by size source of rabies in Europe is raccoon dogs (*Nyctereutes procyonoides)*.

Between 1919 and 1921, rabies was not diagnosed in Lithuania, however, this information is doubtful because soon, in 1922, 22 animals infected with this disease were found. In 1923, the greatest outbursts of rabies were described in Kėdainiai, Raseiniai, Kaunas and Panevėžys districts (Svičiulis and others, 1989). In 1924–1927, over 260 infected animals were registered, in 1928-1931 this figure stood at 288. In 1940-1950, mainly cats and dogs were infected with rabies, and in 1960-1969 rabies cases were established in almost 1500 animals. Domestic animals were infected more often (68%) than wild animals (32%). In 1970–1979, as many as 1333 rabies cases were discovered; the infection rate of domestic and wild animals was almost the same. 1251 rabies cases were described in 1980-1989 (Svičiulis and others, 1989). In 1994–2003, rabies was registered all over Lithuania (Milius and others, 2004). Most often wild and domestic animals were infected with rabies though there were also cases of contraction of the disease among people, especially in Belarus, Ukraine where many domestic animals were infected with the rabies virus (RBE, 2011).

The only and the most effective way of protecting oneself from rabies after an infected or unknown animal has bitten one, is immunoprophylaxis. The sooner vaccination is started the sooner a higher immunity level will be achieved (Toovey et al., 2007). A human being who was in contact with an unknown domestic or wild animal must receive vaccination against rabies as soon as possible and not to discontinue the prescribed course of vaccination. Seeking to avoid a spread of rabies in the population of domestic animals, the latter must be vaccinated prophylactically on the annual basis against rabies. It was in 1897, on the initiative of the physician V. Orlovski, that the first and one of the oldest stations of Paster was established in Vilnius (Svičiulis and others, 1989). Vaccines were made, vaccination against rabies was given, and scientific research was carried out in that station. The main measure seeking to liquidate the spread of rabies in the population of carnivorous wild animals is oral rabies vaccination (ORV) (Cliquet et al., 2010). The first ORV of wild animals in Lithuania was started in 1995–2000. The effect of oral rabies vaccination of foxes has been assessed on the basis of diagnostic investigations determining tetracycline marker in the jaw bone of the hunted foxes (Milius and others, 2004). However, investigations into the efficacy of ORV of wild animals when determining the response to immunisation from blood (RV specific antibodies in blood serum) were not carried out in Lithuania in 1995-2000. In 2001–2005, oral rabies vaccination of foxes was not carried out. Since 2006 ORV has been started to be carried out in Lithuania again using weakened SAD Berne vaccine (Bioveta, Czech Republic), and since 2011 – weakened SAD B19 vaccine (Fuchsoral, Germany).

At the present time cases of rabies are still diagnosed both in Lithuania and its neighbouring countries (Russia, Poland, Belarus and Latvia). Investigations into the rabies epidemiological situation and the efficacy of vaccination in our country will help elucidate tendencies of the spread of the disease and will reduce a threat for people to become infected with rabies.

Objective of work

To elucidate the epidemiological situation of wild and domestic animals in Lithuania, to carry out investigations into the efficiency of rabies diagnostics and immunoprophylaxis.

Tasks of work

1. To assess epidemiological peculiarities of the rabies virus of wild and domestic animals in Lithuania in 2003–2011.
2. To establish geographical peculiarities of the spread of rabies in Lithuania in 2007–2011.
3. To carry out investigations into rabies diagnostics (by means of fluorescent antibody test (FAT) and rabies tissue culture infections test (RTCIT)) and the efficiency of immunoprophylaxis (by means of the Enzyme-linked immunoassay test (ELISA) and rabies virus neutralisation by means of the fluorescent antibody test (FAVN)) in the samples of wild and domestic animals and to compare and assess the methods being employed.
4. To carry out a philogenetic analysis of rabies viruses (RV) of wild and domestic animals in the region of nucleoprotein (N) gene and to establish the geographical type of the circulating RV.

5. To review and assess rabies preventative measures for people, wild and domestic animals.

Scientific innovation and practical significance of work

It has been established that in Lithuania, between 2003 and 2011, among wild animals, red foxes *(Vulpes vulpes)* and raccoon dogs *(Nyctereutes procyonoides)* were main carriers of the rabies virus of wild fauna in nature. It has been established that the boundary delineated by the geographical view of rabies is along the Nemunas River dividing the southern part of Lithuania – on the Polish border and to as somewhat lesser extent along the Neris and Šventoji Rivers dividing the eastern part of Lithuania – on the Latvian border.

The methods that were used to diagnose rabies are noted for specificity and sensitivity. It has been proved that applying simultaneously the direct FAT and RTCIT methods it was possible to ensure a fast and effective determination of RV in the samples of wild and domestic animals under investigation thereby ensuring confirmation of the diagnosis of being infected with RV. During the ORV period between 2007 and 2011, the philogenetic analysis of RV isolates of domestic and wild animals in the sphere of N gene by means of the reverse transcription-polymerase chain reaction was carried out for the first time. The philogenetic analysis of N gene of RV widespread in the population of wild and domestic animals showed that Lithuanian stocks were included in the subgroup of North East Europe.

In 2006-2011, the assessment of the efficacy of ORV in raccoon dogs and red foxes by means of the quantitative ELISA research method determining antibodies specific to the vaccine rabies virus in blood samples was made for the first time. The sensitivity and specificity established by the method showed that the ELISA method was the most suitable for assessing the efficacy of ORV because with the help of this method it was possible to determine the average and low titre even in highly contaminated samples. We recommend this research method to be used to assess the efficacy of ORV of wild fauna.

It has been established that vaccine against rabies used in Lithuania is an effective and safe measure of immunoprophylaxis, which precludes the spread of rabies. The investigations into the dynamics of antibodies specific to the vaccine rabies virus conducted showed that using SAD Berne vaccine the percentage of the efficacy of ORV in wild animals was higher than that when using SAD B19.

Statements to be defended

- From 2003 to 2006, positive cases of rabies were on the increase in the populations of wild and domestic animals in Lithuania because no measures of immunoprophylaxis were applied to wild animals. Between 2006 and 2011, the measures of immunoprophylaxis against rabies applied to wild animals (red foxes and raccoon dogs) stabilised significantly the spread of rabies.
- Methods of diagnostics of rabies used are specific and reliable.
- Measures of immunoprophylaxis used are noted for their profound effect.

Materials and methods

Between 2003 and 2011, the epidemiological situation of rabies in Lithuania was assessed. In 2006–2011, serological blood tests of wild (red foxes, raccoon dogs) and domestic (dogs, cats and ferrets) animals were carried out and the vaccination efficiency was assessed. Wild and domestic animals from all over Lithuania were investigated by the FAT method (n=17747). Since 2005 investigations into the FAT and RTCIT methods of rabies in the populations of racoon dogs (n=1181) and red foxes (n=1587) have been conducted. Since 2007 investigations into identification of the rabies virus (RTCIT) and molecular tests (AT-PGR) for positive rabies samples (n=75) determined by the FAT method have been carried out. In 2006-2011, blood serums for assessing efficacy of rabies vaccination by the FAVN method (OIE 2011) were selected and studied according to the established requirements set to animal health specified in Regulation No. 998/2003 of the European Parliament and the Council (EU), which is applied to non-commercial movement of domestic animals between the countries of the European Union.

The epidemiological analysis of human rabies was made on the basis of observations of rabies cases in 2006-2010 by the Centre for Infectious Diseases and AIDS, which indicated the number of people injured, what animals injured them, the way immunoprophylaxis has been made, how much rabies vaccine and specific immunoglobulin have been used. In 2006-2010, cleaned inactivated rabies vaccine Verorab® (Sanofi Pasteur, France) was used for rabies vaccination of people.

Collection of samples under investigation. Pathological material for rabies FAT, RTCIT and AT-PGR investigations was taken from the brains of wild animals at the laboratories of the State Food and Veterinary Service and at the Pathologic Anatomy and Histology Unit of the National Food and Veterinary Risk Assessment Institute. To elucidate the epidemiological situation of wild and domestic animal, rabies material for laboratory investigations was collected from the whole territory of Lithuania. Samples were taken in the following cases: a) having suspected that the animal was infected with rabies, i.e., when symptoms characteristic of rabies occured (the animal's behaviour was changed, the animal became aggressive, if it suffered from paresis and paralysis, a wild animal came and settled in a homestead, etc.), b) upon a sudden death of an animal, c) upon finding a dead wild or domestic animal. Blood serum for ORV rabies serological tests of wild animals was taken in a very sterile way from the vein of the heart of the hunted animal. The assessment of the efficacy of ORV of raccoon dogs and red foxes in blood serum samples was made after tetracycline in the jaw bone of the hunted animals had been established using it as a biological marker in baits with vaccine intended for ORV of wild animals.

Immunological, virusological and molecular investigations. Diagnostics of rabies was made in accordance with the biological safety requirements at the rabies laboratory (OIE, 2011). The rabies samples of brains under investigation and reference biological substances were prepared at the class II biological safety cabinet of A level (Hera safe, Germany).

Establishment of antigen of the rabies virus by the fluorescent antibody test (FAT). Commercial diagnostic polyclonal antigens (Bioveta, Czech Republic) chemically combined with fluorescein isothiocyanate (FITC) specific to the rabies virus were used for brain sample tests by means of the FAT method. Diagnostics of rabies by the FAT method was made according to the standardised World Organisation for animal Healt

(OIE) and World Health Organization (WHO) (OIE, 2011) requirements. The results of the FAT test of rabies were assessed under the luminescence microscope with x40 objective lens magnification (Nikon, Japan).

Rabies Tissue Culture Infection Test (RTCIT). Identification of RV was made by means of RTCIT infecting N2a CCL-131 cell lines with rabies-positive brain suspension and stained by applying direct FAT. The RTCIT test was carried out on a 96-well plate by pouring 200 µl of cell suspension of $3x10^5$/ml N2a CCL-131 prepared concentration into each well. Following incubation N2a CCL-131 cell lines were inoculated with 100 µl of animal brain supernatant. After incubation the inoculated N2a CCL-131 cells were fixed with 80% acetone, dried and stained with FITC marked monoclonal antibodies specific to the Rabies virus (Fujirebio, USA) according to the manufacturer's instruction. The stained cells were assessed under luminescence inverted microscope with x40 objective lens magnification (Leica, Germany).

Enzyme-linked immunoassay test (ELISA). The commercial ELISA kit Platelia™ Rabies II (Bio-Rad, France) was used to determine the efficacy of ORV against rabies of red foxes and raccoon dogs. The indirect ELISA method was conducted following the instruction of the manufacturer of the diagnostic kit. Measurements were made at the 450nm wave length by means of the automatic micro plate analyser (Elx808, Bio-Tek, USA).

Fluorescent antibody virus neutralisation test (FAVN). The following reference materials were used for FAVN test: BHK-21 C13 (ATCC CCL-10) cell line, the laboratory virus of rabies CVS-11 ATCC VR 959 stock, control OIE positive serum 0,5 TV/ml and negative reference serum. Commercial diagnostic FITC marked monoclonal RV specific antibodies (Fujirabio, USA) were used for direct FAT staining. The FAVN reaction was carried out by means of micromethod using a 96-well plate. Prior to carrying out FAVN reaction the virus of CVS-11 ATCC VR 959 stock was titrated and its titre in the tissue culture BHK-21 C13 amounted to $10^{5,1}$ $TCID_{50}$/ml (50% of tissue culture infection dose). The smallest amount of antibodies of the serum under study ($TCID_{50}$) was regarded as the virus neutralisation titre in which viruses were neutralised in 50% of holes, i.e., when CPE was completely stopped and calculated by the Spearman-Kärber method (Spearman, 1908; Kärber, 1931). Serum titre was expressed in terms of the international units in a millilitre (IU/ml) as compared with reference positive and negative titres obtained in each test.

Assessment of enzyme-linked immunosorbent assay and fluorescent antibody virus neutralisation test. In the course of tests the assessment of the ELISA and standard FAVN ("*gold standard*") methods used to investigate the efficacy of ORV of wild animals against rabies was made. By means of these methods blood serum samples of red foxes (n=50) were examined. The results obtained were compared assessing sensitivity and specificity of reactions according to OIE (OIE, 2011). Specificity of the reaction was defined as the probability that red foxes, having not eaten live attenuated SAD Bern vaccine (established by standard FAVN) reaction) in reality, would show a negative result of the reaction. Sensitivity of the reaction was defined as the probability that red foxes possessing no really formed response to immunisation (established by standard FAVN reaction) would show a positive result.

Establishment of the rabies virus by means of the reverse transcription-polymerase chain reaction (AT-PGR). Extraction of the rabies virus RNR from the brain samples was made with the help of the commercial Rneasy Mini Kit (Qiagen)

following the instruction of the manufacturer of the kit. The AT-PGR reaction was carried out using OneStep RT-PCR kit (Qiagen). PGR reaction mixture was prepared for one test-tube of the reaction consisting of the following: 5µl of AT-PGR buffer, 1µl of dNTP mixture up to 10mM concentration, 1µl of enzyme mixture and 1µl of each of the two primers – JW12 (5'-AT ATGTAACACCYCTACAATG-3') and JW6DPL (5'-CAATTCGCACACATTTTGTG-3') up to 20mM of concentration, 11µl of water and 5µl of RNR under study. Reverse transcription: +50°C 30 min., initial denaturation +95°C 15 min., further during PGR 35 amplification cycles with initial denaturation +94°C 30 min. was made, adhesion of primers +49°C 30 s., DNR synthesis +72°C 1 min., completion of DNR fragments +72°C 10 min. establishment of the amplified 362 bp product was made in 1.5% agarose gel. The products obtained were purified using 500 U SAP (Fermentas, Lithuania) and 10 U ExoI (Fermentas, Lithuania), as well as Centri-Sep biosystems according to the manufacturers' recommendations. Sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing kit according to the instruction of the manufacturers of biosystems (Applied Biosystems 3100 Avant, USA).
Statistical analysis of the data. The statistical analysis of the data of the samples under study was made with the help of the computer SPSS (*Statistical Package for the Social Science*) (16.00) statistical data processing programme. Qualitative indicators were described presenting their percentage distribution. The Chi square test was used to assess statistical reliability of the data and links between the factors being analysed. Differences were statistically significant if the value of the probability of the mistake p was smaller than 0.05. The reliability of difference between the reliability interval and percentage of RV prevalence in the samples of animal titres with 95% of probability was calculated with the help of "Dimension Research", Inc. programme.

Results and discussion

Analysis of prevalence of rabies of wild and domestic animals in Lithuania. Between 2003 and 2011, prevalence of rabies in the populations of wild and domestic animals was registered in all districts of Lithuania. During that period a total of 17747 animals, including 11817 wild and 5930 domestic animals from different districts of Lithuania were investigated (*Fig 1).* In the course of 9 years, out of that number 4860 wild and 1297 domestic animals infected with RV were established. The results obtained in investigating general samples of the animals' brains (*Fig. 1)* showed different infectiousness of RV in the titre samples of wild and domestic animals.

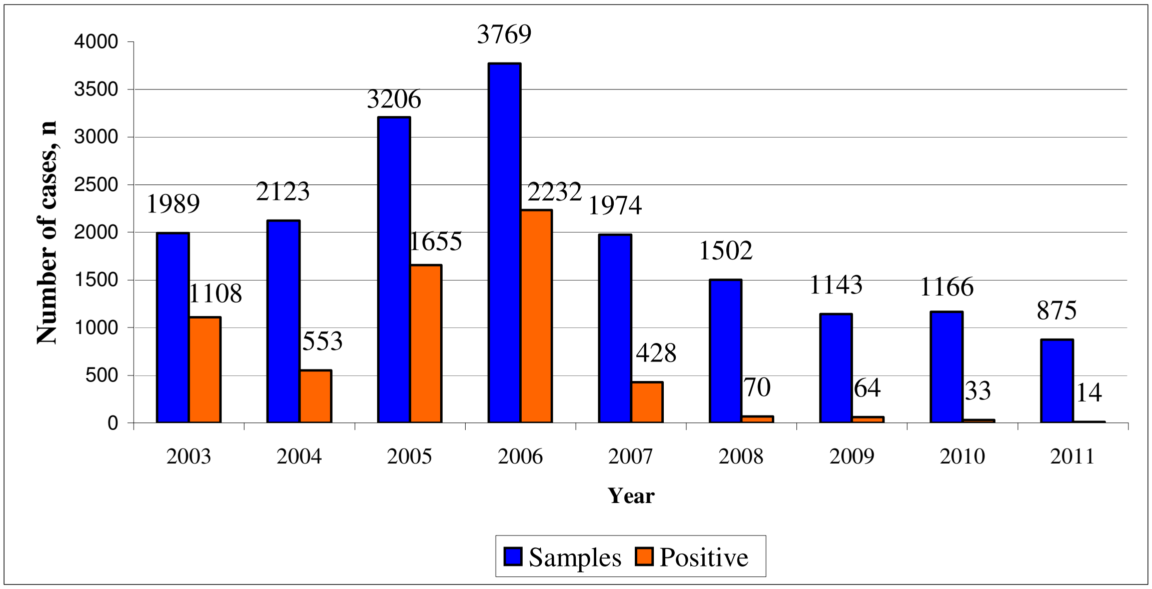


Figure 1. **The number of animal rabies cases (total for all species) in Lithuania during 2003-2011.**

The highest ratio of the animals infected with RV was in the group of wild animals – 41.13% (PI 40.24 % – 42.02%), and that in a group of domestic animals accounted for 21.87% (PI 20.84 % – 22.94%). The statistical analysis of the results made showed that wild animals infected with rabies infected domestic animals with RV because statistically significant increase in RV ($p<0.05$) was noticed. The statistical analysis that we carried out showed that from 2003 to 2011 rabies infectiousness of animal decreased on average by 6.9 percentage points during a year ($p=0,001$) (*Fig. 2*).

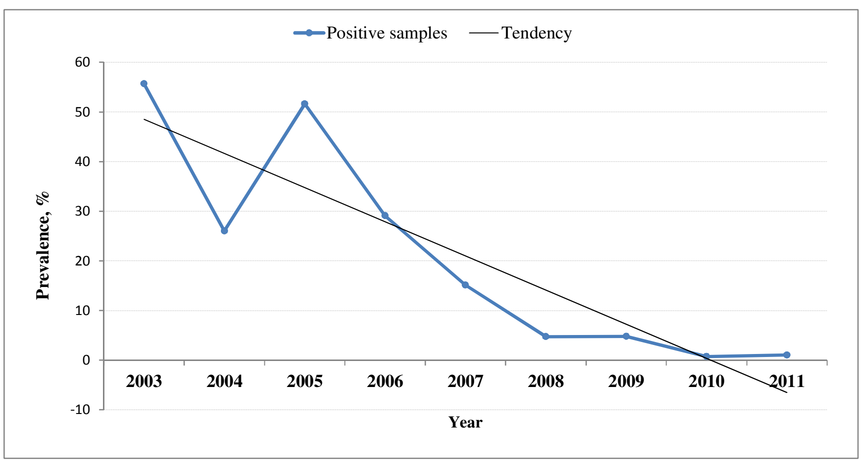


Fig 2. **Dynamics of rabies infectiousness of animals in Lithuania between 2003 and 2011**

Having carried out the analysis of the data of investigations (2003–2011) according to kinds of wild animals it has been established that in the greatest prevalence of the rabies virus in the samples studied was established in raccoon dogs – 53.88% (PI 52.39% – 55.41%), red foxes – 41.10% (PI 39.73% – 42.50%), badgers – 49.33% (PI 38.33% – 60.40%), martens – 30.69% (PI 28.26% – 33.23%), wolves – 28.57% (PI 8.22% – 64.11%), beavers – 10.99% (PI 6.08% – 19.06%), minks – 8.06% (PI 3.49% – 17.53%), otters – 13.95% (PI 6.56% – 27.26%), elks – 5.56% (PI 0.99% – 25.76%), roe-deer – 9.60% (PI 6.54% – 13.89%), wild bores – 3.57% (PI 0.98% – 12.12%), hedgehogs – 4.55 (PI 0.81% – 21.80%), squirrels – 8.70% (PI 2.42% – 26.80%), lynx – 66.67% (PI 20.77% – 93.85%), rats – 2.44% (PI 0.67% – 8.46%). When analysing rabies infectiousness of wild and domestic animals, it was established in the samples that during the period between 2005 and 2011 rabies infectiousness was highest in the populations of wild animals. The highest infectiousness in wild animals was established in 2006 – 73.10% (PI 71.36% – 74.78%, as compared with 2005 – 62.68% (PI 60.59% – 44.72%). Higher RV infectiousness among domestic animals was established, in 2005 – 30.69% (PI 28.04% – 33.46%), and in 2006 it was lower and accounted for 29.10% (PI 26.59% – 31.75%) (*Fig. 3*). The analysis of the data according to the number of samples of wild and domestic animals studied showed that in 2006 the number of positive cases of rabies reached the highest level — 2232 cases, that is, 59.22% (PI 57.64% – 60.78%). Since 2007 the number of positive cases has been on the decrease and in 2011, only 14 animals infected with rabies were established, which accounted for as little as 1.6% (PI 0.96% – 2.67%) of the total number of animals under study (*Fig. 3*).

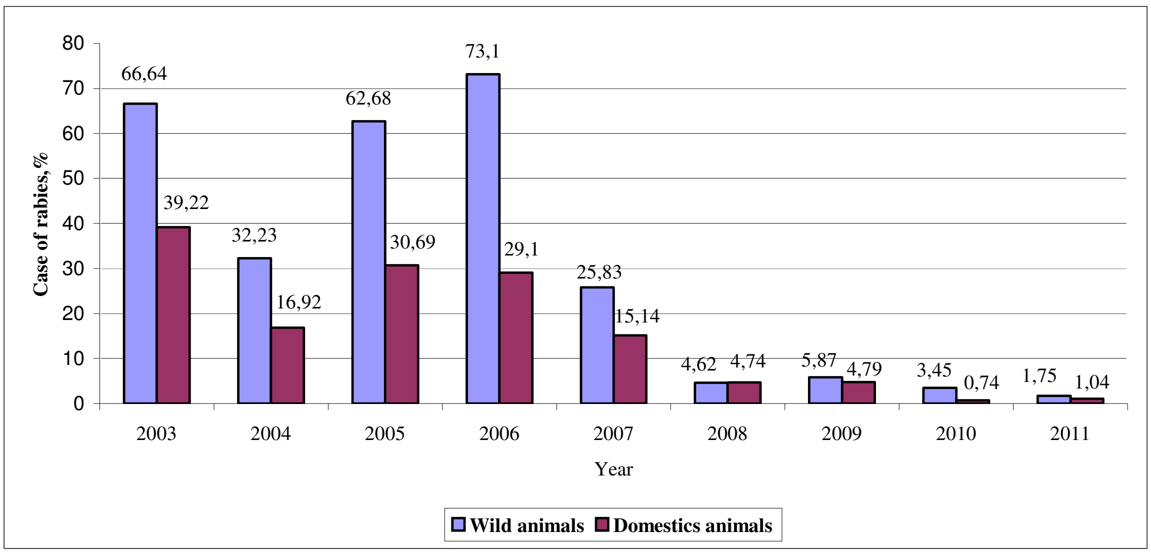


Fig. 3. **Percentage of prevalence of wild and domestic animals infected with rabies virus in Lithuania between 2003 and 2011**

From 2003 to 2011, infectiousness of wild animals with rabies decreased on average by 8.8 percentage points per year (p=0,008). Rabies infectiousness of domestic animals decreased on average by 4.6 percentage points per year (p=0,002) during the same period. The dynamics of infectiousness of both wild and domestic animals was very similar, there was no statistical different between the tendencies in change (p=0,123) (*Fig. 4*).

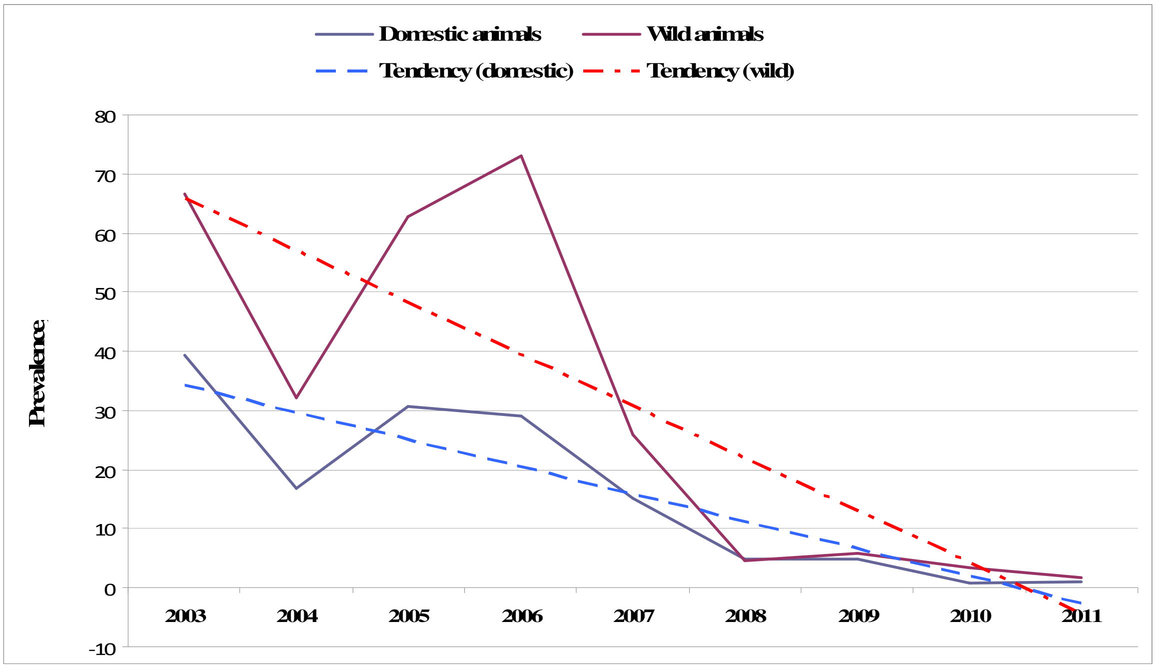


Fig 4. **Dynamics of prevalence of wild and domestic animals infected with rabies in Lithuania between 2003 and 2011.**

From the analysis of the data of investigations into infected wild animals presented in Fig. 5 it can be seen that in 2003-2004, in 2007 the highest infectiousness with the rabies virus in nature was among foxes. In 2004 they accounted for 31.51% (PI 27.98%-35.27%), but in 2005-2006 and in 2008 – 2011 more cases of infectiousness with RV were established in the population of raccoon dogs (*Fig.5).*

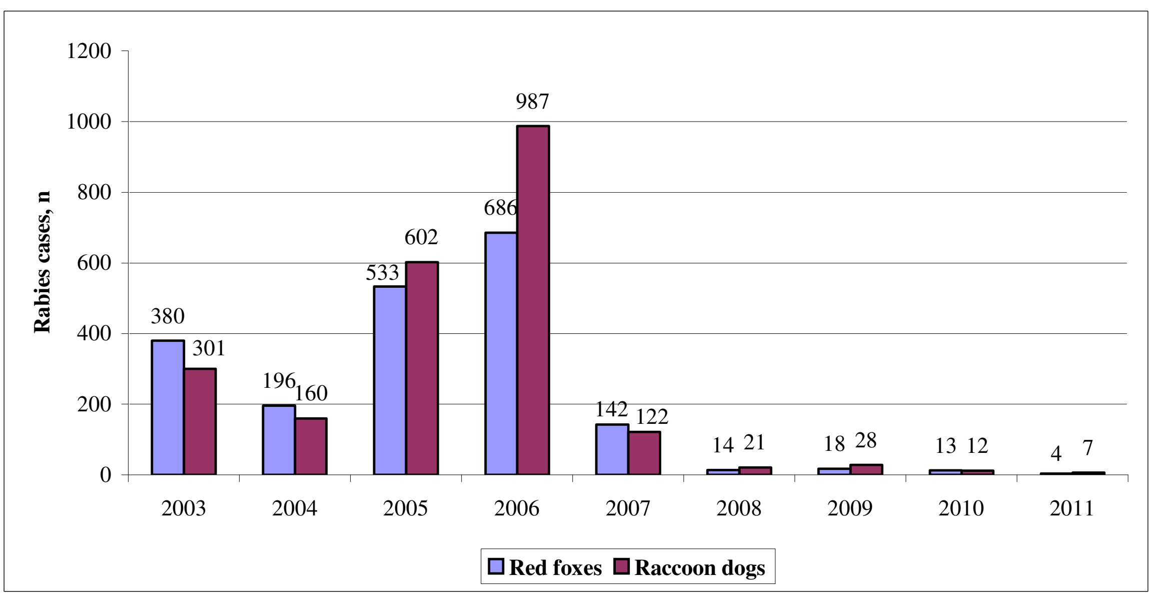


Fig. 5. **Dynamics of infectiousness of red foxes and raccoon dogs in Lithuania between 2005 and 2011.**

A generalised analysis of the rabies FAT results showed that wild (foxes, raccoon dogs) and domestic carnivorous animals (dogs, cats) were the main source of rabies infection between 2003 and 2011 therefore a risk for other wild animals (roe-deer, elks, hares, wild bores and others) and domestic animals (cow, horses, goats and others) to become infected was increased. Scientists of other countries noticed the same, that is, the epidemiological condition of rabies was mainly related to an increase in the population of racoon dogs and foxes. With rabies viruses infecting the populations of raccoon dogs and red foxes the number of individuals therein started to decrease to the threshold concentration of susceptible animals per area unit ensuring persistent transmission of rabies (Toma, Andral, 1977).

Geographical peculiarities of rabies prevalence. Having assessed the dynamics of rabies infectiousness of wild and domestic animals during a five-year period (2007–2011), decreasing numbers of rabies cases of wild and domestic animals have been established *(Table 1).*

Table 1. Rabies infectiousness in wild and domestic animals in Lithuania between 2007 and 2011.

Year	Wild animals			Domestic animals			p*
	Examined, n	Positive, %	PI, %	Examined, n	Positive, %	PI, %	
2007	1208	25.83	23.36 – 28.30	766	15.14	12.60 - 17.68	0.0001
2008	1038	4.62	3.34 - 5.90	464	4.74	2.81- 6.67	0.9208
2009	851	5.88	4.30 - 7.46	292	4.79	2.34 - 7.24	0.4882
2010	897	3.46	2.26 - 4.66	269	0.74	-0.28 - 1.76	0.0186
2011	684	1.75	0.77 - 2.73	191	1.05	-0.40 - 2.50	0.4910

*p is difference in comparing wild and domestic animals (Chi test).

The analysis of 2007 – 2011 carried out showed that during the period under study the highest infectiousness of both wild and domestic animals was established in 2007 (almost 26% of wild and 15% of domestic animals); this difference between domestic and wild animals was statistically significant ($p<0.05$). In 2008 – 2009, a decrease in infectiousness among both wild and domestic animals was observed; no statistically significant difference was established that year ($p>0.05$). In 2010, the number of both wild and domestic infected animals was somewhat larger and this difference was statistically significant ($p<0.05$). In 2011, no significant difference between wild and domestic animals was established.

Between 2007 and 2011, the largest number of rabies cases was established on the Lithuanian-Belarus border, that is, in the eastern part of Lithuania, in those districts, which border with neighbouring Republic of Belarus – in Švenčionys, Šalčininkai, Varėna, Zarasai. The map of Lithuania (*Fig. 6*) shows a geographical distribution of rabies cases of different species of animals studied and the exact location from which the sample was taken. We think that rivers can be a natural barrier in the territory of Lithuania and that the border delineated by a geographical view can be along the Nemunas River, which separates the southern part of Lithuania – on the border with Poland and to a lesser extent, along the Neris and Šventoji Rivers, which separate the eastern part of Lithuania – on the border with Belarus (*Fig. 6).*

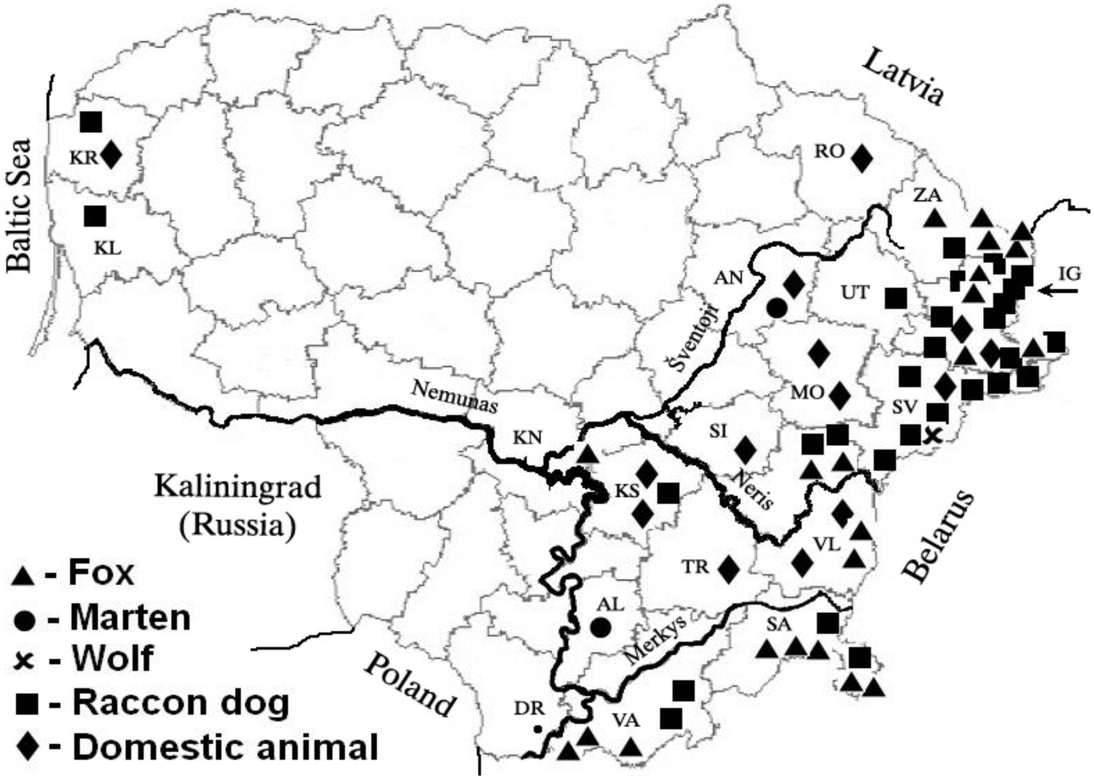


Fig. 6. **The geographical distribution of rabies in different species of animals in Lithuania between 2007 and 2011.**

Abbreviations of districts: Alytus-AL, Anykščiai-AN, Druskininkai-DR, Ignalina-IG, Kaunas-KN, Kaišiadorys-KS, Kretinga-KR, Molėtai-MO, Rokiškis-RO, Šalčininkai–SA, Širvintos-SI, Švenčionys-SV, Varėna-VA, Vilnius-VL, Trakai-TR, Zarasai-ZA.

It can be stated that the rabies virus could have got into our country from neighbouring Belarus in which vaccination of wild animals against rabies was not carried out until 2011. On the basis of rabies FAT results it can be stated that having given ORV to wild animals the rabies cases was annually on the decrease. However, our epidemiological investigations showed that despite ORV that has been applied thus far, 14 cases of rabies were registered in eastern districts of Lithuania in 2011, which bordered on Belarus, which confirmed our statement that the rabies virus could have come to our country from neighbouring Belarus.

Meantime the highest rabies infectiousness was established in Lithuania's neighbouring countries in 2006: 1499 cases in Belarus, 1349 cases in Russia, 472 cases in Latvia (RBE, 2011). Comparing the number of recorded rabies cases with that in the neighbouring countries at the same time (2006) the largest number of rabies cases was established in Estonia (Cliquet et al., 2012). During the period between 2007 and 2011, the number of positive cases of rabies was on the decrease. If 428 rabies cases were recorded in 2007, in 2011 only 14 animals infected with rabies were regcorded, which accounted for as little as 1.6% of thetal number of all animals under investigation.

In summing up the rabies cases recorded n 2007-2011 it can be stated that a decrease in rabies cases was observed not only in Lithuania but also in the hbouring countries, with the exception of Belarus (RBE, 2011).

Diagnostic studies of rabies of wild animals. Investigations into rabies of wild animals carried out in Lithuania between 2003 and 2011 showed that the main source of rabies is raccoon dogs and red foxes. A total of 4159 brain samples of raccoon dogs were studied by means of FAT between 2003 and 2011. Our investigations (2003-2011) showed that rabies FAT positive cases in the samples of raccoon dogs under study accounted for 53.88% (PI 52.39% – 55.41%). On the basis of the investigations carried out in 2003 – 2004 a tendency for rabies cases to decrease was observed – in 2003 74.14% (PI 69.84% – 78.36%), and in 2004 – 41.13% (PI 36.21% – 45.99%, $p<0.05$ (chi test) (*Fig. 7*).

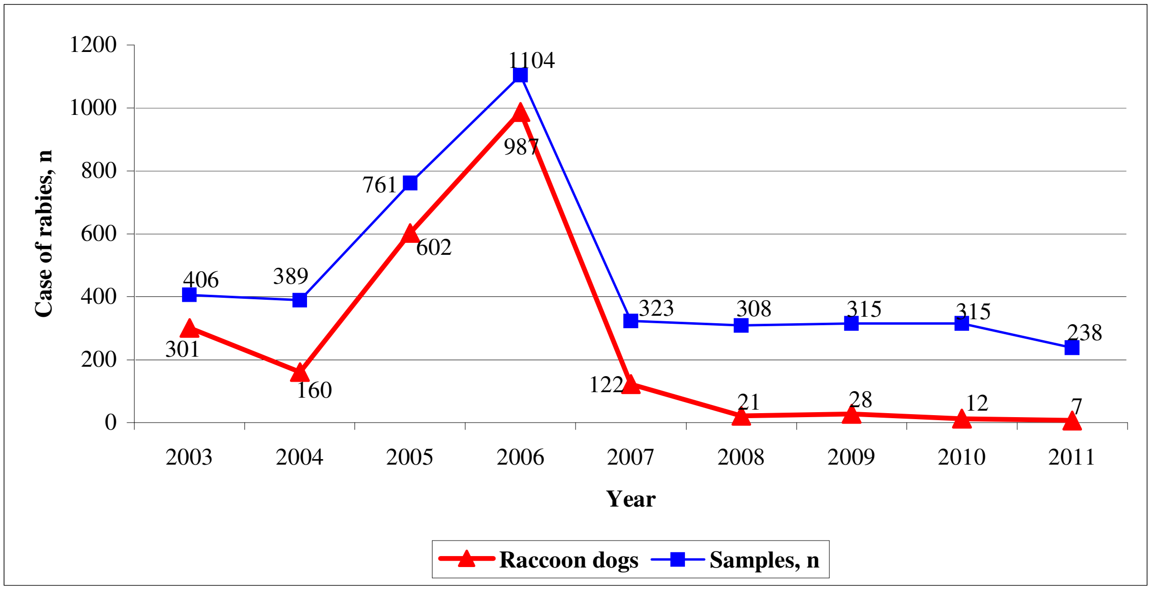


Fig.7. **Dynamics of rabies infectiousness of raccoon dogs in Lithuania between 2003 and 2011.**

Having made the analysis of the data according to the year of carrying out the investigation, it was established that the highest infectiousness of rabies of raccoon dogs was in 2005 79.11% (PI 76.21% – 81.99%), in 2006 – 89.40% (PI 87.58% – 91.22%). However, in analysing rabies cases of raccoon dogs recorded in 2007 it was established that the incidence of rabies decreased to 37.77% (PI 32.51% – 43.09%), in 2008 – 6.81% (PI 3.99% – 9.61%), in 2009 – 8.89% (PI 5.76% – 12.04%), in 2010 – 4.13% (PI 1.91% – 6.29%). In 2011, the lowest infectiousness of raccoon dogs was established – 2.94% (PI 0.77% – 5.03%). From 2003 to 2011, cases of rabies in raccoon dogs decreased on average by 10.3 percentage points per year, this tendency was statistically significant ($p=0.009$).

The assessment was made on the basis of joined investigations carried out with other authors comparing differences in prevalence of rabies in the populations of raccoon dogs. The difference was as follows: the highest prevelence of the raccoon dog population was observed between 2002 and 2003, and significantly lower prevalence was observed in 2004. In 2005-2006, an increase in the population prevelance was established (Robardet, Cliquet, 2010). According to our data, the largest number of rabies cases of raccoon dogs was also recorded in 2006 (n=987) (*Fig. 7*). During the period between 2007 and 2011, it was established that the number of recorded rabies cases in the population of raccoon dogs was on the decrease every year. This could have been influenced not only by ORV of wild animals that was started but also by the equal number of racoon dogs in the population in Lithuania established by the data of other investigators in 2007-2009.

When analysing the epidemiological situation of red foxes, during the period between 2003 and 2011, a total of 4834 brain samples of red foxes was studied by FAT method in Lithuania. Our investigations (2003-2011) showed that rabies FAT positive cases in the samples of red foxes under study accounted for 41.10% (PI 39.73% – 42.50). As compared with 2003 and 2004, a tendency towards a decrease in rabies was observed – in 2003 – 74.07% (PI 70.28% – 77.87%,), and in 2004 – 31.51% (PI 27.86% – 35.16%, p<0.05 (chi test). However, in 2005 and 2006, an increase in rabies cases was observed again: in 2005 – 63.91% (PI 60.59% – 67.10%), and in 2006, the highest infectiousness of red foxes in the course of the entire period between 2003 and 2011 was observed – 79.95% (PI 77.14% – 82.50%) (*Fig.8*). When further analysing rabies cases of red foxes recorded in 2007 it was established that incidence was again on the decrease –32.20% (PI 27.84% – 36.56%), and in 2008 – 3.29% (PI 1.60% – 4.99%), in 2009 – 5.10% (PI 2.80% –7.39%), in 2010 – 3.10% (PI 1.50% – 4.70%). In 2011, the lowest infectiousness of red foxes was established – 1.19% (PI 0.03% – 2.34%) of cases (*Fig. 8*).

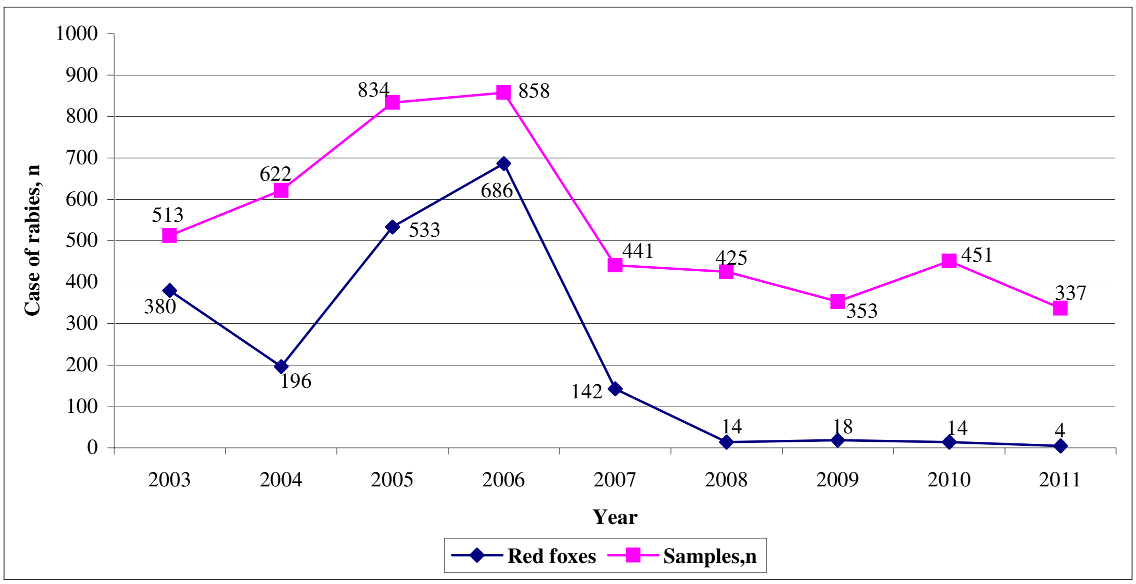


Fig. 8. **Dynamics of rabies infectiousness of red foxes in Lithuania between 2003 and 2011**

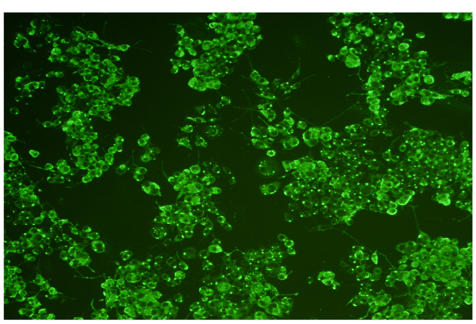
In summing up it can be stated that from 2003 to 2011, incidences of rabies in red foxes had a tendency to decrease and on average decreased by 9.5 percentage points per year, this tendency was statistically significant (p=0.009).

According to the assessment made by other scientists, when comparing distribution of the population of red foxes between 2003 and 2007, in 2004 a considerably smaller distribution of the populations was established (Robardet, Cliquet, 2010). According to the data of these authors, the distribution of the population of red foxes was on the increase until 2007-2009. However, in 2007-2009, the distribution of the population remained unchanged (Robardet, Cliquet, 2010). Our epidemiological investigations carried out showed a growth in the population of red foxes therefore we can state that an increase in the rabies cases established by us (n=686) could have been influenced by an increase in the population of these wild animals (*Fig.8*). In 2007-2011, we established that the number of recorded rabies cases was on the decrease each year and this decrease could have been influenced not only by rabies oral vaccination of these animals but also by the fact that an increase in the population of red foxes in 2007-2009 was not observed.

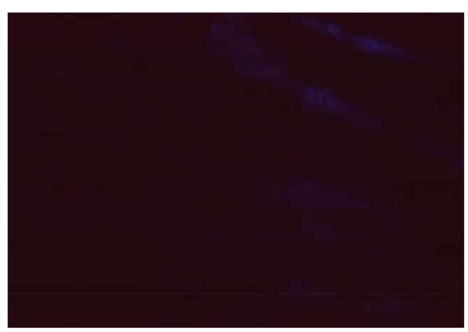
The investigations into rabies of wild animals carried out in Lithuania showed that the main source of rabies was racoon dogs and red foxes. The results of these investigations corresponded with the data obtained by other investigators studying prevalence of rabies cases in the populations of racoon dogs and red foxes in three Baltic States (Lithuania, Latvia and Estonia) between 1995 and 2009 (Robardet, Cliquet, 2010). It is stated in those investigations that from 1989 to 2009, the largest number of rabies cases 7249 (48%) in the three Baltic States was discovered in the population of red foxes (n=15101), out of the same number of 15101 samples examined in the population of raccoon dogs 5906 (39%) positive cases were found (Robardet and Cliquet, 2010). It was only in November of one year, i.e., in 2000, that a tendency for rabies to be prevalent in the population of racoon dogs was observed. The analysis of the investigators' results showed that in 2000, a larger number of rabies cases in the population of raccoon dogs than that in the population of red foxes was established in Estonia and Lithuania (Robardet and Cliquet, 2010).

Assessment of the method of establishing virus antigen by fluorescent antibodies and rabies tissue culture infections tests. The comparative RV FAT used in the investigation into the samples of the distribution of raccoon dogs and foxes under study, and the assessment of RTCIT methods was made by confirming animals infected with RV by each method.

The same brain samples were used for comparing FAT and RTCIT methods of raccoon dogs. By means of the FAT method a total of 1181 brain samples of raccoon dogs were studied. Rabies was established in 115 – 9.74% (PI 8.17% - 11.56%) of them. Having examined the same 1181 brain samples of raccoon dogs by the RTCIT method, identical results were obtained, RV was isolated (*Fig. 9)*. In the same way the same brain samples of red foxes were used for investigations to compare FAT and RTCIT methods. A total of 1587 brain samples of red foxes were examined by the FAT methods and rabies was discovered in 66 – 4.16% (PI 3.28% – 5.26%,) in them. Having examined the same brain samples by means of the RTCIT method the same results were obtained, RV was isolated.



a) Positive result.



b) Negative result.

Fig 9. **Identification of rabies virus in N2a CCL-131 tissue culture by means of direct FAT method.** When assessing by means of the RTCIT method, granules of different size located inside a cytoplasm of the cell infected with RV fluorescent – shine in bright green apple colour. The result is positive when having assessed the entire area of the well we discovered one or more fluorescenting cells (*Fig. 9 a)*. The result is negative when having assessed the entire area of the well not a single fuorescenting cell was discovered (*Fig.9 b*).

The analysis of the data of our investigations showed that statistically equal number of raccoon dogs and red foxes infected with RV was established by means of both FAT and RTCI methods. The methods that we applied to diagnose rabies proved specificity and reliability of the methods. It is pointed out in literature that reliability of the results obtained by the FAT method was 95–99% if the sample was fresh and delivered within several hours for examination (OIE, 2008). Sensitivity of the FAT method can be affected by the brain autolysis; also, *Lyssa* kind of virus and a too small amount of the brain or perhaps the sample that has been taken from a vaccinated animal can influence the results too (OIE, 2008).

The following RV specific antibodies to mark FITC are used to diagnose rabies by means of the FAT method in the EU countries – to stain the brain tissue: polyclonal antigens (Bioveta), monoclonal antibodies (Biorad), monoclonal antibodies (Fujireb), monoclonal antibodies (Millipore), monoclonal antibodies (SIFIN) (Robardet et al., 2012). These five above-mentioned diagnostic preparations were used to carry out a quantitative and qualitative analysis and it was established that FITC marked antibodies of Fujireb Company were the most sensitive and were one of five best diagnostic preparations and were the most suitable for diagnosing rabies. The Millipore diagnostic preparation was the most suitable to establish the virus of EBLV-1 kind; also the diagnostic preparation of Fujireb Company equaled it and it was used to establish viruses of EBLV-2 and ABLV kind (Robardet et al., 2012). These results showed that the efficiency was related to specific antibodies, monoclonal antibodies used showed the results of higher compliance (Robardet et al., 2012). The analysis of preparations for diagnosing rabies carried out in co-operation with the laboratories of other countries once again confirmed that rabies diagnostics carried out by us was reliable because the results of FGAT tests were confirmed by the RTCIT and AT-PGR methods of rabies virus identification in tissue culture.

Assessment of rabies serological research methods. The same number of blood samples of red foxes was examined by means of the FAVN and ELISA methods. A total of 50 samples of blood serum of red foxes was examined by the ELISA research method and in 28 samples antibodies were discovered. The same 50 blood serum samples of red foxes were examined by the FAVN method, and in 23 of them antibodies were found (*Table 2).*

Table 2. Distribution of the antibody research data in age groups of red foxes depending on the diagnostic method applied.

Red foxes group	Samples, n	Positive samples, n				p*
		FAVN		ELISA		
		n	%	n	%	
< 1 year	25	8	32.00	10	40.00	0.5557
> 1 year	25	15	56.00	18	72.00	0.3705
Total	50	23	46.00	28	56.00	0.3172

*p is difference in comparing FAVN and ELISA (Chi test).

A different number of serologically positive samples of red foxes was established by the FAVN and ELISA methods. When examining them by means of the ELISA method, more serologically positive red foxes (56%) were found than when examining them by means of the standard FAVN method (46%), however, this difference was not statistically significant. Though these differences statistically were not significant, in practice such differences in research results could have an impact on the quality of investigations therefore seeking to elucidate differences in the test results of blood serum obtained by means of the standard FAVN and ELISA, we assessed ELISA sensitivity and specificity according to the calculation schemes recommended by OIE.

ELISA showed a very high diagnostic sensitivity and specificity exceeding 95%. Having compared the ELISA and FAVN methods discrepancies in the results obtained in the samples where titres of antibodies were low were established: the titre of a blood sample of one red fox was 0.38 TV/ml near 0.5 TV/ml, therefore it was classified as negative. The greatest amount of discrepancies was due to the cytotoxic blood serum samples of red foxes, which suppressed the growth of cells in carrying out the FAVN reaction but cytotoxicity of the samples was not important in carrying out tests by means of the ELISA method. Since all blood serum samples collected were taken from the hunted foxes, this discrepancy was not of great significance to diagnostics of specific vaccine RV antibodies. The analysis of the research results showed that the ELISA methods that we employed was sensitive and did not produce any non-specific reaction due to a poor quality of the sample. Therefore to assess the fficacy of ORV of raccoon dogs and red foxes we chose the ELISA research method. The ELISA commercial kit contained plates covered with rabies virus glycoprotein ready for use and by means of simple and fast technique of carrying out ELISA the amount of rabies antibodies in the samples of wild fauna could be established (Mebatsion et al., 1989, Esterhuysen et al., 1995; Cliquet et al., 2000, 2003, 2004, 2007; Servat et al., 2007). Scientists from other countries also conducted studies to determine suitability of the ELISA method and in some cases their opinions differed therefore different scientific discussions were still being held on this issue, also, new ELISA kits of different manufacturers were being tested (Cliquet et al, 2000, Servat et al. 2007, Knoop et al. 2010, Wasniewski, Cliquet, 2012).

Assessment of the efficacy of rabies oral vaccination of raccoon dogs and red foxes. In carrying out investigations into humoral immunity of raccoon dogs and red foxes we applied the ELISA method on the basis of which we made the analysis of the data of antibodies specific to vaccine RV. A total of 4122 samples of the hunted raccoon dogs and red foxes were studied between 2006 and 2011. Our investigations showed that antibodies specific to vaccine RV were determined in 2185 blood samples, and the efficacy of ORV was 53.01% (PI 51.48 % – 54.53%). In analysing blood serum tests of raccoon dogs it was established that antibodies specific to vaccine RV accounted for 48.64% (PI 45.87% – 51.41%). The analysis of the data between different age groups of raccoon dogs (up to one year of age and adults > 1 year) showed different ORV efficacy (chi test, p=0,004). Blood samples of 229 raccoon dogs (up to one year of age) were studied. Specific antibodies were found in 87 samples (37.99%, PI 31.70% – 44.28%). Antibodies were found in 523 out of 1025 blood samples of older raccoon dogs studied (51.02%, PI 47.96% – 54.08%) (*Table 4*).

Table 4. Serological investigations into the efficacy of rabies oral vaccination of raccoon dogs and red foxes between 2006 and 2011.

Year (month)	**The age groups**	**Raccoon dogs**			**Red foxes**		
		Samples, n	Positive, %	PI, %	Samples, n	Positive, %	PI, %
2006 (06-12) 2007 (01-10)	Cubs <1 year	25	8.0	-2.63-18.63	61	29.51	19.56-41.89
	Adults >1 year	90	41.11	30.94-51.28	429	51.28	46.55 – 56.01
2007 (10-12)	Cubs <1 year	31	22.8	7.86-37.30	26	34.62	16.33 – 52.91
	Adults >1 year	198	55.56	48.64 -62.48	136	41.91	33.62 – 50.20
2008 (01-12)	Cubs <1 year	18	61.11	38.59 -83.63	49	59.18	45.42 – 72.94
	Adults >1 year	149	60.40	52.55 -68.25	464	63.15	58.76 – 67.54
2009 (01-12)	Cubs <1 year	5	40.00	-2.94 -82.94	22	45.45	24.64 - 66,26
	Adults >1 year	49	36.73	23.23 -50.23	214	53.27	46.59 – 59.95
2010 (01-12)	Cubs <1 year	126	44.44	35.76 -53.12	125	66.40	58.12 – 74.68
	Adults >1 year	375	53.33	48,28 -58,38	538	65.99	61.99 – 69.99
2011 (01-12)	Cubs <1 year	24	37.50	18.13 -56.87	99	42.42	32.68 – 52.16
	Adults >1 year	164	41.46	33.92 -49.00	705	48.94	45.25 – 52.63
Total		1254	48.64	45.87-51.41	2868	54.92	53.10- 56.74

In analysing blood serum tests of red foxes it was established that the efficacy of rabies ORV accounted for 54.92% (PI 53.10% – 56.74%) in that population. Blood samples of 382 red foxes (up to one year of age) were examined. Antibodies specific to vaccine RV 50.00% (PI 44.99% – 55.01%) were found in 191 samples (*Table 4*). Specific antibodies in 2486 samples of older red foxes were found in 1384 samples (55.67%, PI 53.72% – 57.62).

The analysis of the data on the efficacy of ORV showed that similarly to the difference in different age groups of raccoon dogs (up to one year of age and adults > 1 year), there was also statistically significant difference in red foxes of different age groups (p=0,0381). It can be stated that formation of immunity against the rabies virus more often was more effective among the older than younger animals.

With immunity of the population strengthening, the population becomes more abundant, i.e., the number of animals susceptible to infection increases, especially in the groups of the young during the summer (Niin et al., 2008). In Lithuanian ORV of wild animals is carried out twice a year – in spring and autumn. Raccoon dogs have their young in May in Europe because their pregnancy period is longer than that of foxes (Kauhala, 1996), and foxes have their young beginning with April (Llyod, 1980). Differences in the way of taking food between foxes and raccoon dogs are also observed: cubs of foxes take food (bait) with their teeth (Mac Donald, 1987), and those of raccoon dogs do not take food (Llyod, 1980). This is confirmed by the results of our investigations – a lower efficacy of ORV was established among the young of raccoon dogs than that among their adults. Therefore it should be noted that successful ORV of wild animals depends on several factors, i.e., a clear location of vaccination, the time of vaccination, the way the vaccine (food) was distributed and its amount, properties of the vaccine and the efficiency of the programme.

Phylogenetic analysis of the rabies virus. ORV of wild animals that has been carried out in Lithuania since 2006 has reduced considerably cases of rabies. However, between 2007 and 2011, when a tendency for the increase in the epidemiological process of rabies of wild and domestic animals on the Lithuanian-Belarusian border was observed, phytogenetic investigations of RV nucleoprotein (N) were carried out. Between 2007 and 2011, by means of the FAT and RTCIT methods 75 brain cases positive to rabies were selected, including 59 wild and 16 domestic animals from different districts of Lithuania: red foxes (n=25), raccoon dogs (n=31), wolves (n=1), martens (n=1), badgers (n=1), dogs (n=7), cats (n=4) and cattle (n=5). The tests conducted by means of the AT-PGR method confirmed that all 75 samples contained RV.

Calculations were made with the help of "Clustal W" programme, the comparison of sequences made and a percentage identity of nucleotide sequences calculated showed that the rabies virus samples of wild and domestic animals collected in Lithuania in 2007-2011 were genetically close (97-100%) (*Fig 10*). Sequences of viruses circulating in neighbouring countries were selected for the geographical comparison of sequences (Kissi et al., 1995; Vanaga et al., 2003; Mansfield et al., 2006; Larkin et al., 2007; Zienius et al., 2009). It was established that isolates of rabies samples of red foxes in the sphere of N gene were also phylogenetically close similarly to those of raccoon dogs, the wolf, the marten, dogs, cats and the cattle. The same RV stocks of Belarus, Poland, Estonia, Latvia and Russia were used for investigations. Nucleotide sequences of RV subgroups common in Europe and presented in the database "GenBank" were used for the phylogenetic analysis and the construction of a tree (Kissi et al., 1995; Vanaga et al., 2003; Mansfield et al., 2006; Larkin et al., 2007; Zienius et al., 2009).

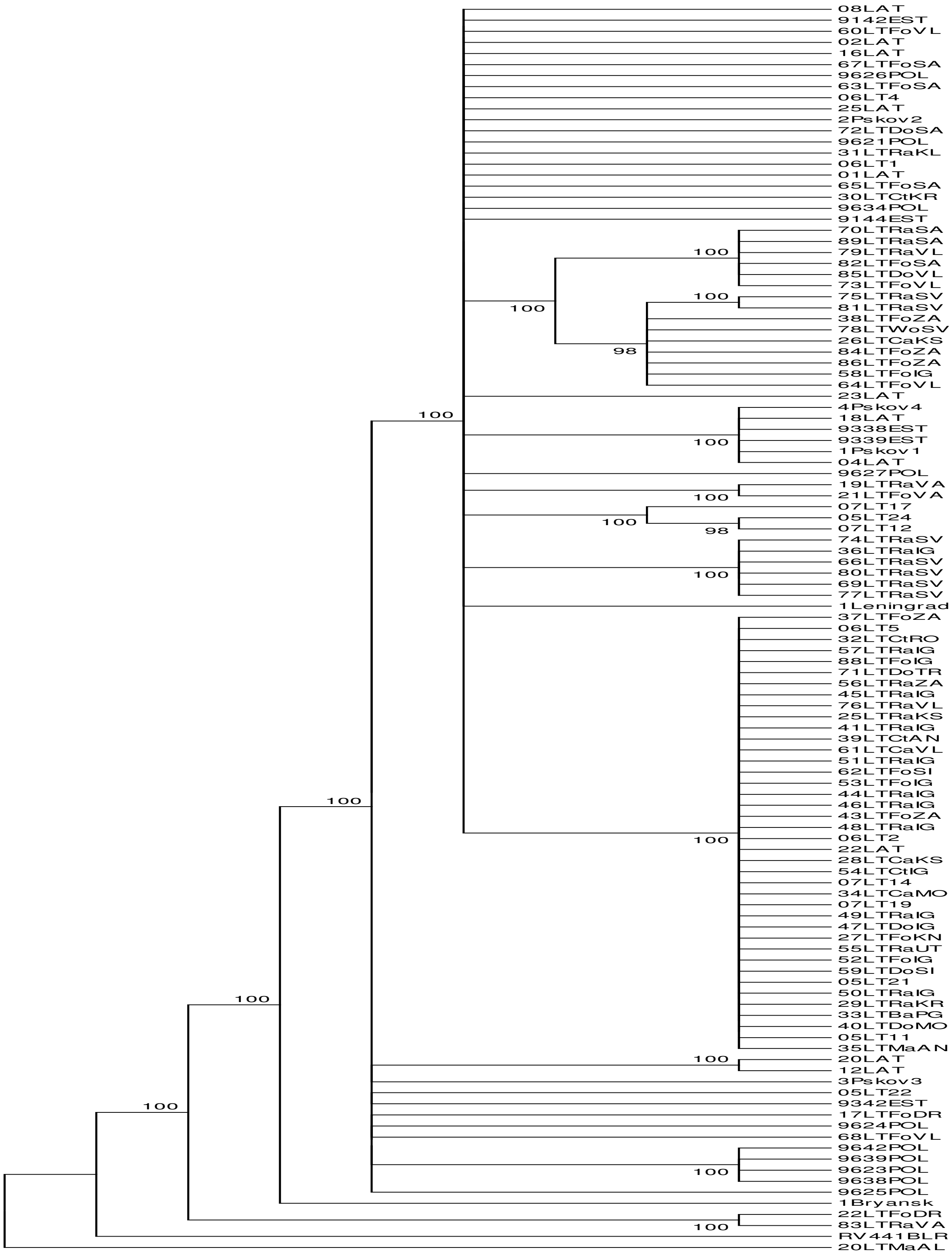


Fig 10. **Phylogenetic tree: Rabies virus detection and phylogenetic analysis in samples from wild and domestic animals of Lithuania in 2007-2010.** Phylogenetic analysis of RV N gene 362bp cirulating in the Lithuanian populations of wild and domestic animals. Maximum parsimony trees were drown using "ClustalW" aligned sequences and MEGA4 package. Statistically significant 70% cutoff was chosen for consensus trees. Abbreviations: LTFoSA – Lithuania fox Šalčininkai, LT – Lithuania; animal species: Cat – Ca, Cattle – Ct, Dog – Do, Fox – Fo, Marten – Ma, Raccoon dog – Ra, Wolf – Wo; districts: Alytus – AL, Anykščiai – AN, Druskininkai – DR, Ignalina – IG, Kaunas – KN, Kaišiadorys – KS, Kretinga – KR, Molėtai – MO,Rokiškis – RO, Šalčininkai – SA, Širvintos – SI, Švenčionys – SV, Utena – UT, Varėna – VA, Vilnius – VL, Trakai – TR, Zarasai – ZA)

Having analysed the results of our investigations it was established that RV stocks of wild and domestic animals in N gene sequence, together with the RV stocks of neighbouring countries, formed clearly defined philogenetic branches in phylogenetic trees. Our phylogenetic analysis of N gene 362 bp confirmed the conclusions of the investigations described by other authors that all rabies viruses of Lithuania belonged to the first genotype and were attributed to North East European – NEE subgroup (McElhinney et al., 2008, Zienius et al., 2009) irrespective of the date of isolation of the rabies virus (samples were collected in 2007-2010). RV stocks of the neighbouring countries were grouped together with the Lithuanian ones without indicating either specific or clear geographical adherence thereby confirming earlier observations. Insignificant differences in nucleotides allowed the conclusion to be made that rabies viruses were not absolutely homogeneous in the populations of red foxes or raccoon dogs and other wild or domestic animals. On the basis of phylogenetic analysis it was impossible to determine exactly if red foxes were the source of the rabies virus and infected raccoon dogs or visa versa, later raccoon dogs infected foxes (Bourhy et al., 1992, 1993).

In summing up our investigations carried out in Lithuania between 2007 and 2011 during the ORV period, it can be stated that a phylogenetic analysis of N gene sequences showed a close direct geographical link between the neighbouring countries: Estonia, Latvia, Belarus and Poland. Other scientists also noticed a close direct geographical link between these countries (Kissi et al., 1995; Vanaga et al., 2003; Mansfield et al., 2006; Larkin et al., 2007; Zienius et al., 2009).

Analysis of immunoprophylactic measures On the basis of statistical data prevalence and vaccination of rabies in the populations of people, domestic and wild animals in Lithuania was studied**.** During the period under study (between 1960 and 2010) 12 cases of people's deaths from rabies were recorded. Dogs and foxes were the most frequent source of infections (*Table 5*). In 2007, a positive rabies case of a person was diagnosed in Lithuania. The person was not vaccinated against rabies and while travelling in India he was bitten by a dog.

Table 5. Reported cases of human rabies in Lithunia in 1960-2010.

Region	Years	Number of cases, n	Source of infection
Vilnius city	1960	1	Dog
Kaišiadoriai district	1962	1	Fox
Švenčioniai district	1965	1	Raccoon dog
Kėdainiai district	1972	1	Badger
Trakai district	1979	1	Fox
Joniškis district	1992	1	Raccoon dog
Trakai district	1992	1	Dog
Trakai district	1993	1	Cat, dog
Kėdainiai district	1997	1	Fox
Pasvalys district	2000	1	Fox (Raccoon dog?)
Prienai district	2004	1	Not found
Vilnius city	2007	1	Dog (in the territory of India)

When analysing the data of 2006-2010 reports of the Centre for Infectious Diseases and Aids it was established that during the past five years up to 7-10 thousand people suffered from animals in Lithuania every year. Most often people were injured by dogs (70–80%) (*Table 6*). In 2006–2009 – 23–30%, and in 2010 only 1.92% people fell victim to rabid dogs.

Table 6. Distribution of exposed persons according to the source animal species in 2006-2010.

Source of infection	**Year**				
	2006	**2007**	**2008**	**2009**	**2010**
Dogs	7152	6562	5821	5430	5535
Cats	1458	1293	1139	1054	1111
Rats	63	74	70	58	38
Cattle	550	212	187	50	18
Other domestic animlas	175	46	66	85	67
Other wild animals	1392	404	168	246	211
Total	10790	8591	7451	6923	6980

Improperly kept, stray dogs and dogs that had their owners bit 20,485 adults and 10,015 children. Rabid dogs injured 911 persons. The latter received necessary medical help and were vaccinated against rabies. 6055 persons sought medical help because they were bitten or scratched by cats. 461 persons out of that number were injured by rabid cats, and 3314 persons were injured by healthy cats, and 2279 people suffered injuries from the unknown cats. Country people most often are injured by the cattle or their young. It was established that 1017 country people sought medical help between 2006 and 2010 and 844 of them were injured by the cattle infected with rabies, and 146 adults suffered from injuries inflicted by healthy cattle, and 27 people were injured by the unknown cattle. People who fell victim to animals in Lithuania between 2006 and 2010 were vaccinated with the inactivated rabies vaccine Verorab® (Sanofi Pasteur, France) and injected with specific immunoglobulin Imogam®Rabies (Sanofi Pasteur, France) or Favirab (Sanofi Pasteur, France) (*Fig.11*).

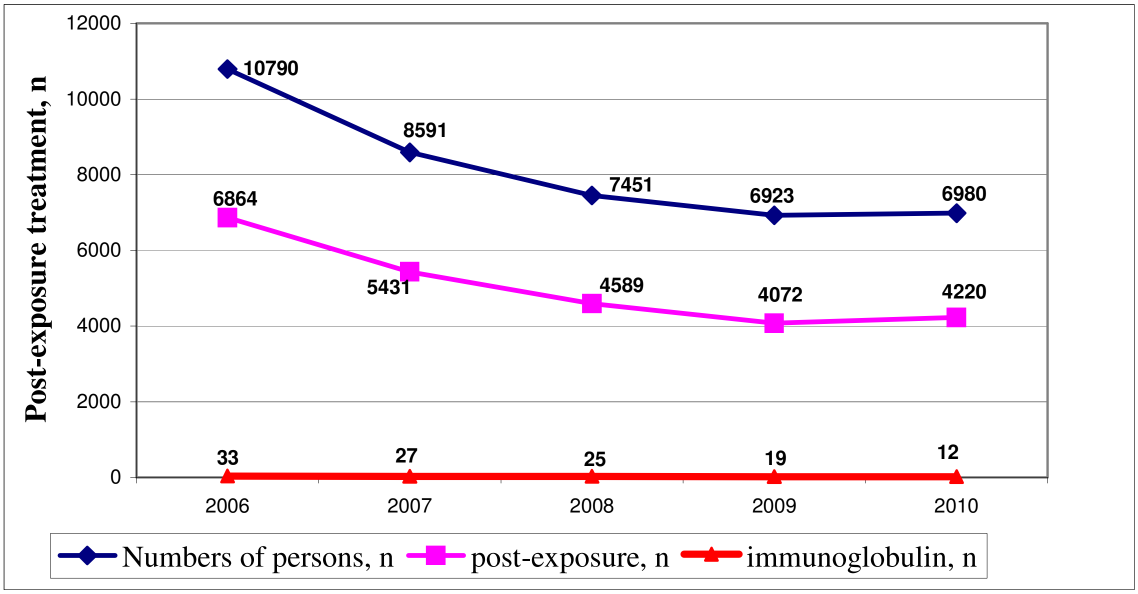


Fig. 11. **Dynamics of exposed and vaccinated persons in 2006-2010.**

Since 2006, ORV of wild animals using a live attenuated SAD Berne vaccine has been carried out in Lithuania twice a year – in spring and autumn, and since 2011 – using the live attenuated vaccine SAD B19. The analysis of two live attenuated rabies vaccines used in ORV was made. It was established that SAD Berne and SAD B19 in the number of seropositive animals in wild animal species differed significantly: SAD Berne of raccoon dogs (p=0.0222) was 50.00% (PI 47.00% – 53.00%), and SAD B19 – 40.96% (PI 33.93% – 47.99%); SAD Berne of red foxes (p=0,001) was 57.56% (PI 55.42% – 59.68%) and SAD B19 – 48.13% (PI 44.68% – 51.58%) (*Fig. 12*).

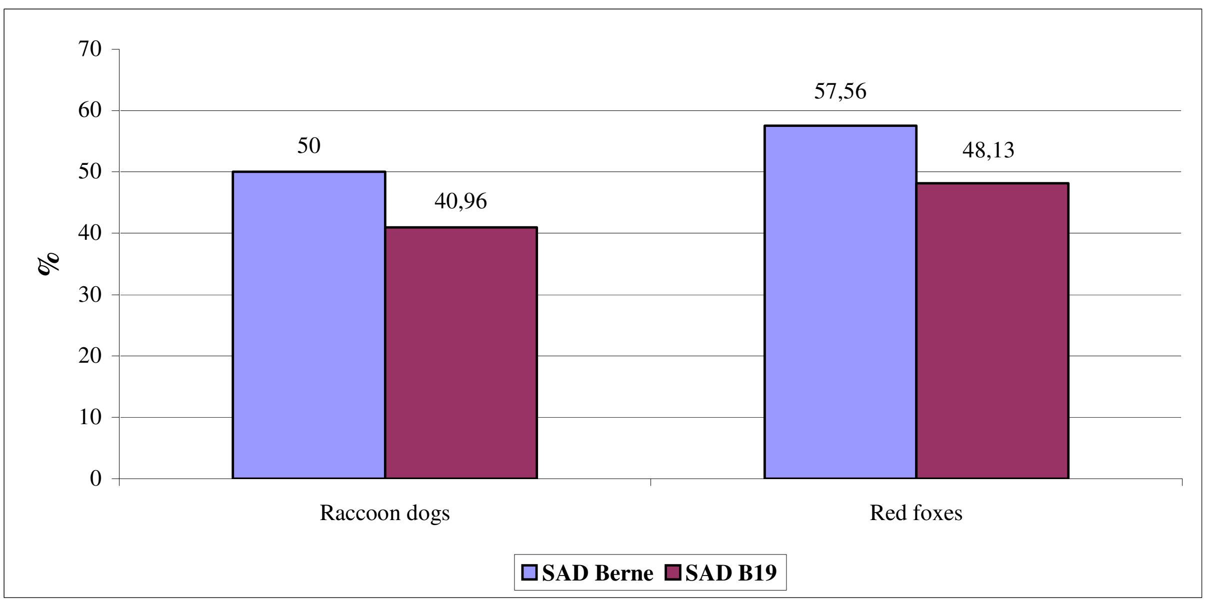


Fig. 12. **Dynamics of the efficacy of wakened rabies viruse vaccines of SAD Berne and SAD B19 stock**

Studies of the dynamics of the antibodies specific to the vaccine rabies virus carried out showed that using the SAD Berne vaccine the percentage of the efficacy of ORV of wild animals was higher than that when using SAD B19 (*Fig. 12*). The efficacy of ORV depends to a great extent on the stability of viruses and the bait. In spring and summer, the amount of viruses in the vaccine decreases rapidly. The bait decomposes rapidly when the ambient temperature is high or when it rains (EC, 2002); division of baits in wet weather can be ineffective in carrying out ORV. Between 1992 and 2000, during the ORV programme in Latvia, 443710 baits were distributed in the territory covering an area of 4000 km^2 and between 2001 and 2003, ORV was continued to be carried out in the entire territory (Vanaga et al., 2003). Since 2005 the programme has been carried out in Latvia in spring and autumn distributing baits together with the vaccine from a plane, the density being 23-25 baits/km^2. During the said period SAD Berne and SAD B19 vaccines were used in Latvia and since 2009 – only SAD B19. Between 2005 and 2011, in carrying out oral immunisation of wild animals in Estonia, rabies was successfully irradiated. Since 2008 only 4 cases of rabies were diagnosed on the south-eastern border of the country (Cliquet et al., 2012). SAG-2 vaccine (Rabigenh, Virbac Laboratories, Carros, France) (Cliquet et al., 2012) was used for oral immunisation of foxes and raccoon dogs in Estonia. In 2011, no cases of rabies were recorded in Estonia, and only one case of rabies was recordered in Latvia (Cliquet et al., 2012). These data presented by the scientists of Lithuania and other countries confirm, our conclusion that the vaccines used in ORV of wild animals in Lithuania are sufficiently effective.

Seeking to put a stop to the spread of the rabies virus among animals compulsory vaccination of domestic animals (cats and dogs) against rabies was carried out in Lithuania between 2006 and 2011. When analysing the research results it was established that dogs constituted the largest number of vaccinated animals – 88,7927, cats – 18,940, the cattle – 25,4490, horses – 4757, sheep and goats – 2261, and other domestic animals – 2186**.** All other domestic animals were vaccinated following their contact with the infected animals or animals which were suspected of having been infected with rabies in the nidi of this disease (*Figure 7*).

Table 7. Vaccinated domestic animals against rabies in Lithuania 2006 – 2011.

Year	**Dogs**	**Cats**	**Cattle**	**Horses**	**Sheep and goats**	**Other domestic animals**
2006	154470	32418	68459	1527	620	600
2007	141880	29475	55586	1094	644	114
2008	163202	35185	49367	850	383	552
2009	152558	33348	33452	632	430	310
2010	141884	29748	26821	391	119	307
2011	133933	29306	20805	263	65	303

In 2006**–**2011, FAVN tests were conducted. In assessing the FAVN results it was established that 5106 domestic animals (dogs, cats and ferrets) were examined and antibodies specific to vaccine RV >0.5TV/ml in titre, which accounted for 94.73% (PI 94.08% – 95.31%) were established in 4837 of them (*Fig. 13*).

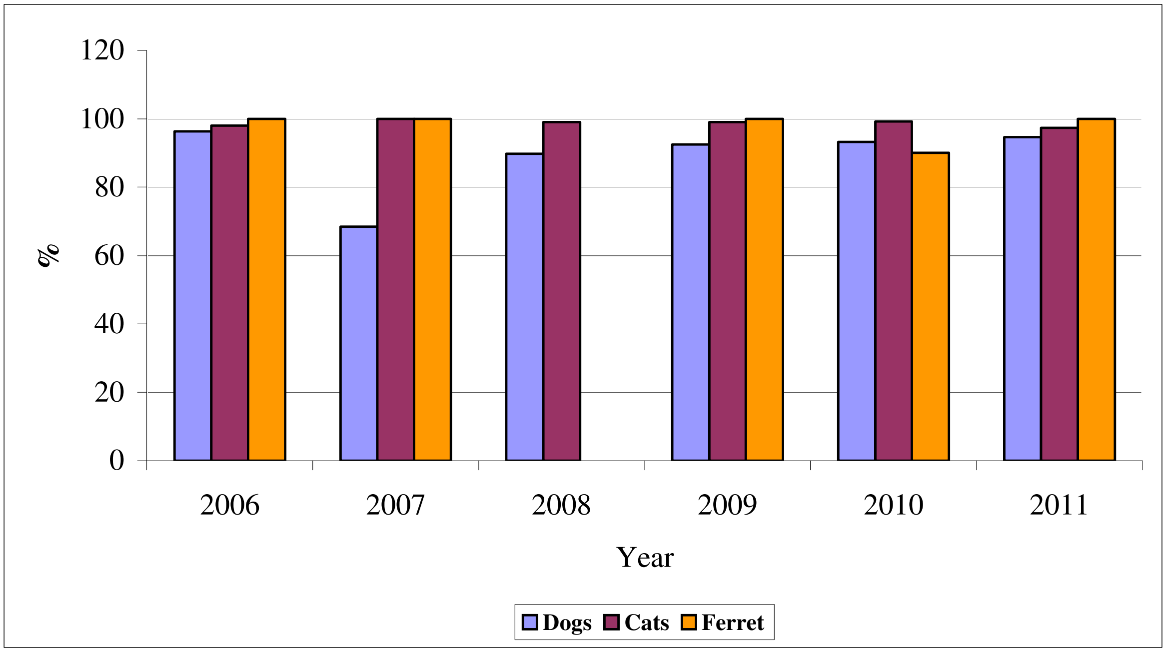


Fig. 13. **Serological investigation into the efficacy of rabies vaccination of domestic animals between 2006 and 2011.**

Having made the analysis of the research data according to the kinds of domestic animals the following antibodies specific to vaccine RV were established: for dogs 93.86% (PI 93.08% – 94.55%), cats 98.52% (PI 97.52 %– 99.11%) and domestic ferrets 96.43% (PI 82.29% – 99.37%). Having applied a linear regression model to the data of the efficacy of vaccination of domestic animals against rabies between 2006 and 2011, it can be stated that the efficacy has a tendency for a decrease (on average 0.52 percentage point per year, p=0,4610), however, this decrease is not statistically significant. Hence, the conclusion can be drawn that the efficacy of vacciantion of domestic animals against rabies during the period under study was stable and amounted to 95%. According to the data on the efficacy of rabies vaccination of dogs between 2006 and 2011, it can be stated that this indicator remains stable during the period under discussion, though it has an insignificant tendency to decrease (on average 0.62 percentage point per year, p=0,8046). Exactly the same conclusion can be drawn when investigating the data on the efficacy of vaccination of cats against rabies: though it has a slighter tendency for a decrease than the efficacy of dogs (on average 0.13 percentage point per year, p=0,6573), it can also be stated that this indicator during the period under discussion remains stable. Having summed up the FAVN research data among different kinds of domestic anaimals it can be stated that vaccines used for immunoprophylaxis of rabies of domestic animals in Lithuania are effective.

It can be stated that a large number of cases of rabies in Lithuania in 2006 had a direct influence on epidemiology of human rabies because that year a large number of people suffered from domestic animals, especially from dogs and cats. This number has been on the decrease since 2007; however, it still remains large. Therefore, in our opinion, the control of the population of stray dogs and cats must be tightened, work in society about how to behave with unknown and stray animals and what dangers are hidden in it should be carried on and intensified. It is only by using immunoprophylactic measures that it is possible to block the way to people contracting rabies. Verorab®

vaccine used against rabies in Lithuania is highly effective and its reactogenicity is incomparably lower than that of the earlier used vaccines. According to the data of the WHO, using this vaccine in 100 countries no undesirable symptoms have been noticed in the course of 20 years (Toovey, 2007). A person who had contact with an unknown domestic or wild animal must as soon as possible become vaccinated against rabies and not discontinue the prescribed vaccine course. However, on the basis of the 2006-2010 results of vaccination against rabies, the conclusion can be drawn that there is a large number of people who discontinue the prescribed course of vaccination.

Having summed up the 2006-2010 results of immunoprophylaxis against rabies, we established that Lithuanian people, and especially animals, are not safe yet though fewer and fewer positive cases of rabies are recorded every year. Annually recorded cases of rabies on the Belarusian frontier are really disconcerting. Modern rabies vaccines used in Lithuania at the present time are not especially effective or safe therefore when the slightest suspicion about contracting rabies infection arises it is necessary to start post-exposure vaccination. We can state that ORV of wild animals being carried out gives positive results in fighting with this dangerous infectious disease contracted from animals. However, in our opinion, perhaps applying animal rabies immunoprophylactic measures should be more intensive in those districts, in the frontier with Belarus districts, in which nidi of rabies are still established at the present time.

Conclusions

1. When analysing the epidemiological situation of rabies of wild and domestic animals between 2003 and 2011, it was established that the dynamics of rabies infectiousness of both wild and domestic animals was very similar, there was no statistical difference between tendencies of change, and rabies infectiousness of animals in the samples studied decreased on average by 6.9% per year.
2. In carrying out the analysis of geographical distribution of rabies between 2007 and 2011, it was established that during the period under study the high infectiousness of both wild and domestic animals was established in the eastern and south-eastern parts of Lithuania. It was established that nidi of rabies were still being recorded in the districts of Švenčionys, Šalčininkai, Varėna, Zarasai, which border on the neighbouring Republic of Belarus therefore it is necessary to take into account application of immunoprophylactic measures in our neighbouring countries.
3. In carrying out diagnostics of rabies by means of the FAVN it was established that from 2003 to 2011, raccoon dogs were the main carriers of the rabies virus. On the basis of the data of the statistical analysis, incidence of rabies decreased on average by 10.3% per year, and that of red foxes – by 9.5% per year.
4. Studies into the methods of rabies fluorescent antibodies and identification of the rabies virus in tissue cultures showed high specificity and reliability of the methods. By means of both methods a similar number of samples of raccoon dogs and red foxes infected with rabies was established.
5. Having studied Enzyme-linked immunoassay and standard fluorescent antibody virus neutralisation tests it was established that Enzyme-linked immunoassay test kit was noted for high sensitivity (95.83%) and specificity (96.15%). The quality of the sample had no impact on the research results in the immunoenzyme analysis therefore this method is recommended to be used in the studies of the efficacy of oral vaccination against rabies.

6. Enzyme-linked immunoassay tests show a sufficient protective efficacy of the vaccines to raccoon dogs (48.64%) and red foxes (54.92%). It was established that building of immunity against the rabies virus was reliably more effective among older than younger animals. The established efficacy of rabies vaccination of domestic animals (dogs, cats and ferrets) during the period under study was stable and accounted for about 95%.
7. Having made a phylogenetic analysis of the pathogenic stocks of the rabies virus of wild and domestic animals during the period of oral vaccination (2006-2011) according to nucleotide sequences of the changing part of N gene it was established that Lithuanian rabies virus stocks are included in North East European group.
8. In carrying out the analysis of two live attenuated rabies vaccines SAD Berne and SAD B19 for rabies oral vaccination of wild animals it was established that the number of seropositive animals in the species of wild animals differed significantly. It was established that using the SAD Berne vaccine the percentage of the efficacy of ORV of wild animals (54.98%) was higher than that when using SAD B19 (43.77%).
9. Having assessed the rabies immunoprophylactic preventative measures that were taken between 2006 and 2011, it was established that Lithuanian people, and especially animals, are not safe yet though fewer and fewer positive rabies cases are recorded every year.

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4. Report delivered at the seminar organised by the State Veterinary Centre of Belarus. **Jacevičienė I.** "Pasiutligės laboratorinė diagnostika". (Laboratory Diagnostics of Rabies) "Pasiutligės profilaktika ir diagnostika" (Prophylaxis and Diagnostics of Rabies), Gardin, Belarus. 2009.
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Reziumė

Pasiutligė yra virusinė liga – viena iš seniausių infekcijų, pavojinga žmonių ir gyvūnų gyvybei. Ligą sukelia RNR turintis virusas, esantis *Mononegavirales* eilėje, priklausantis *Rhabdoviridae* šeimai, *Lyssavirus* genčiai. Pasiutligės viruso (RV) daugiausia randama sergančių laukinių ir naminių gyvūnų taip pat ir žmonių galvos smegenyse, daug - stuburo smegenyse, seilių liaukose, seilėse. Šia liga užsikrečiama kai pasiutlige sergantis gyvūnas įkanda kitam gyvūnui ar žmogui, ar apseilėja sužalotą odą. Šiuo metu yra žinoma, kad atsiradus simptomams, liga paprastai nepagydoma, todėl vienintelis apsisaugojimo būdas nuo pasiutligės, apkandžiojus sergančiam ar nežinomam gyvūnui, yra imunoprofilaktika.

Šiuo metu pasiutligė vis dar yra nustatoma tiek Lietuvoje, tiek ir mūsų kaimyninėse šalyse (Rusija, Lenkija, Baltarusija ir Latvija). Nustatyta, kad Europoje, daugiausia (85 proc.) teigiamų pasiutligės atvejų sudaro rudosios lapės *(Vulpes vulpes),* kurios yra vienos iš pagrindinių pasiutligės viruso platintojų Europoje (Aubert et al., 1995), o antrasis pagal dydį pasiutligės šaltinis yra usūriniai šunys (*Nyctereutes procyonoides*). Pastaruoju metu pagrindinė priemonė siekiant likviduoti pasiutligės paplitimą mėsėdžių laukinių gyvūnų populiacijoje yra oralinė vakcinacija. Nuo 2006 metų Lietuvoje buvo pradėta laukinių gyvūnų oralinė vakcinacija (ORV) naudojant gyvą nusilpnintą SAD Berne vakciną (Bioveta, Čekijos Respublika), o nuo 2011 metų gyvą nusilpnintą SAD B19 vakciną (Fuchsoral, Vokietija). Darbo metu buvo atlikti laukinių ir naminių gyvūnų pasiutligės diagnostiniai imunologiniai, virusologiniai ir molekuliniai tyrimo metodai. Tokio pobūdžio tyrimai svarbūs mūsų šaliai, aiškinantis laukinių ir naminių gyvūnų pasiutligės epidemiologinę situaciją ir vakcinacijos efektyvumą bei pavojingos ligos plitimo tendencijas.

Analizuojant 2003-2011 metų laukinių ir naminių gyvūnų pasiutligės epidemiologinę situaciją Lietuvoje nustatyta, kad tiek laukinių, tiek naminių gyvūnų pasiutligės infekuotumo dinamika yra labai panaši, statistinio skirtumo tarp kitimo tendencijų nėra (p=0,123), o gyvūnų pasiutligės infekuotumas tirtuose mėginuose vidutiniškai mažėjo po 6,9% per metus. Mūsų tyrimų duomenimis nuo 2003 iki 2011 metų laukinių gyvūnų pasiutligės infekuotumas vidutiniškai mažėjo po 8,8 procentinius punktus per metus (p=0,008), o naminių gyvūnų pasiutligės infekuotumas vidutiniškai mažėjo po 4,6 procentinius punktus per metus (p=0,002). Nustatyta, kad 2003–2011 metais laukinių gyvūnų tarpe Lietuvoje usūriniai šunys *(Nyctereutes procyonoides)* ir rudosios lapės *(Vulpes vulpes)* yra pagrindiniai laukinės faunos pasiutligės viruso nešiotojai gamtoje. Mūsų atlikti tyrimai (2003-2011 m.) parodė, kad pasiutligės FAT teigiami atvejai usūrinių šunų tirtuose mėginiuose sudarė 53,88% (PI 52,39% – 55,41%). Atlikta tyrimų duomenų analizė pagal tyrimų atlikimo metus parodė, kad didžiausias usūrinių šunų infekuotumas pasiutlige buvo 2005 metais 79,11% (PI 76,21% – 81,99%) ir 2006 metais – 89,40% (PI 87,58% – 91,22%). Tačiau analizuojant 2007 metais registruotus usūrinių šunų pasiutligės atvejus nustatyta, kad susirgimų sumažėjo iki 37,77% (PI 32,51% – 43,09%), 2008 – 6,81% (PI 3,99% – 9,61%), 2009 – 8,89% (PI 5,76% – 12,04%), 2010 – 4,13% (PI 1,91% – 6,29%). 2011 metais buvo nustatytas mažiausias usūrinių šunų infekuotumas – 2,94% (PI 0,77% – 5,03%), susirgimų. Nuo 2003 iki 2011 metų usūrinių šunų susirgimai pasiutlige vidutiniškai mažėjo po 10,3 procentinių punktų per metus, ši tendencija buvo statistiškai reikšminga (p=0,009). Atliekant mūsų tyrimų analizę (2003-2011 m.) nustatyta, kad pasiutligės

FAT teigiami atvejai rudųjų lapių tirtuose mėginiuose sudarė 41,10% (PI 39,73% – 42,50). Lyginant 2003 ir 2004 metus buvo stebima pasiutligės mažėjimo tendencija – 2003 metais 74,07% (PI 70,28% – 77,87%,), o 2004 – 31,51% (PI 27,86% – 35,16%, $p<0,05$ (Chi testas). Tačiau 2005 ir 2006 metais vėl buvo stebimas pasiutligės didėjimas: 2005 metais 63,91% (PI 60,59% – 67,10%), o 2006 metais buvo stebimas didžiausias rudųjų lapių infekuotumas pasiutlige per visą 2003-2011 metų laikotarpį – 79,95% (PI 77,14% – 82,50%) (*8 pav.*). Toliau analizuojant 2007 metais registruotus rudųjų lapių pasiutligės atvejus nustatyta, kad susirgimų vėl mažėjo – 32,20% (PI 27,84% – 36,56%), o 2008 – 3,29% (PI 1,60% – 4,99%), 2009 – 5,10% (PI 2,80% – 7,39%), 2010 – 3,10% (PI 1,50% – 4,70%). 2011 metais buvo nustatytas mažiausias rudųjų lapių infekuotumas – 1,19% (PI 0,03% – 2,34%) susirgimų. Apibendrinant galima teigti, kad nuo 2003 iki 2011 metų rudųjų lapių susirgimai pasiutlige turėjo tendenciją mažėti ir vidutiniškai mažėjo po 9,5 procentinių punktų per metus, ši tendencija buvo statistiškai reikšminga ($p=0,009$).

Atliekant 2007 – 2011 metų pasiutligės geografinio paplitimo analizę buvo nustatyta, kad nagrinėjamu laikotarpiu daugiausia pasiutligės atvejų buvo nustatyta Baltarusijos pasienyje su Lietuva, t.y. rytiniame Lietuvos pakraštyje, tuose rajonuose, kurie ribojasi su kaimynine Baltarusijos Respublika – Švenčionių, Šalčininkų, Varėnos, Zarasų. Mūsų atlikta 2007 – 2011 metų analizė parodė, kad nagrinėjamu laikotarpiu didžiausias tiek laukinių, tiek naminių gyvūnų infekuotumas buvo nustatytas 2007 metais (laukinių beveik 26%, naminių - 15%), šis skirtumas tarp naminių ir laukinių gyvūnų buvo statistiškai reikšmingas ($p<0,05$). 2008 – 2009 metais buvo stebimas infekuotumo mažėjimas tiek laukinių, tiek naminių gyvūnų tarpe, statistiškai reikšmingo skirtumo tais metais nenustatyta ($p>0,05$). 2010 metais laukinių infekuotų gyvūnų buvo šiek tiek daugiau nei naminių, ir šis skirtumas buvo statistiškai reikšmingas ($p<0,05$). 2011 metais reikšmingų skirtumo tarp laukinių ir naminių gyvūnų nenustatyta.

Mūsų naudoti pasiutligės diagnostikos metodai pasižymi specifiškumu ir jautrumu. Nustatyta, kad kartu taikant tiesioginį FAT ir RTCIT metodus, galima užtikrinti greitą ir efektyvų RV nustatymą laukinių ir naminių gyvūnų tiriamuosiuose mėginiuose, tuo užtikrinant diagnozės patvirtinimą dėl užsikrėtimo RV. ORV periodu 2007-2011 metais pirmą kartą buvo atlikta naminių ir laukinių gyvūnų RV izoliatų filogenetinė analizė N geno srityje atvirkštinės transkripcijos polimerazės grandininės reakcijos (AT-PGR) metodu. Laukinių ir naminių gyvūnų populiacijoje paplitusio RV N geno filogenetinė analizė parodė, kad lietuviškos padermės patenka į Šiaurės Rytų Europos pogrupį.

Atlikus imunofermentinės analizės (IFA) ir standartinio viruso neutralizacijos fluorescuojančiais antikūnais (FAVN) metodų tyrimus nustatyta, kad komercinis IFA rinkinys pasižymi dideliu jautrumu (95,83%) ir specifiškumu (96,15%). Nustatytas IFA metodo jautrumas ir specifiškumas parodė, kad IFA metodas yra tinkamiausias ORV efektyvumui įvertinti, nes šiuo metodu galima nustatyti vidutinius ir žemus titrus net ir labai užterštuose mėginiuose. Šį tyrimo metodą rekomenduojame naudoti laukinės faunos ORV efektyvumo įvertinimui.

Pirmą kartą 2006–2011 m. atliktas usūrinių šunų ir rudųjų lapių ORV efektyvumo vertinimas kiekybiniu IFA tyrimo metodu, nustatant specifinius vakcininiam RV antikūnus kraujo mėginiuose. Imunoprofilaktikos tyrimai rodo pakankamą vakcinų apsauginį efektyvumą usūriniams šunims (53,01%) ir rudosioms lapėms (54,92%). Atlikta ORV efektyvumo duomenų analizė parodė, kad kaip ir tarp skirtingų usūrinių šunų amžiaus grupių (iki vienerių metų ir suaugusių > 1metų) taip ir tarp skirtingų amžiaus rudųjų lapių grupių yra statistiškai reikšmingas skirtumas (p=0,0381). Galime teigti, kad imuniteto susidarymas prieš RV dažniau buvo efektyvesnis tarp vyresnio amžiaus gyvūnų nei jaunesnių. Taip pat mes atlikome naminių gyvūnų (šunų, kačių ir šaškų) vakcinacijos nuo pasiutligės efektyvumo vertinimą kiekybiniu FAVN metodu. Vertinant duomenų analizę pagal naminių gyvūnų rūšis buvo nustatyti specifiniai vakcininiam RV antikūnai: šunims 93,86% (PI 93,08% – 94,55%), katėms 98,52% (PI 97,52 %– 99,11%) ir naminiams šeškams 96,43% (PI 82,29% – 99,37%). Pritaikius tiesinės regresijos modelį 2006–2011 m. naminių gyvūnų vakcinacijos nuo pasiutligės efektyvumo duomenimis, galima teigti, kad efektyvumas turi tendenciją mažėti (vidutiniškai po 0,52 procentinio punkto per metus, p=0,4610), tačiau šis mažėjimas nėra statistiškai reikšmingas. Taigi galime daryti išvadą, kad naminių gyvūnų vakcinacijos nuo pasiutligės efektyvumas nagrinėjamame laikotarpyje buvo stabilus ir siekė 95%. Nustatyta, kad Lietuvoje naudojamos vakcinos nuo pasiutligės – efektyvi ir saugi imunoprofilaktikos priemonė, stabdanti pasiutligės plitimą.

Atlikta ORV naudotų dviejų nusilpnintų pasiutligės vakcinų efektyvumo analizė parodė, kad SAD Berne ir SAD B19 laukinių gyvūnų rūšyse seroteigiamų gyvūnų skaičius reikšmingai skyrėsi: usūrinių šunų (p=0,0222) SAD Berne buvo 50,00% (PI 47,00% – 53,00%), o SAD B19 - 40,96% (PI 33,93% – 47,99%); rudųjų lapių (p=0,001) SAD Berne buvo 57,56% (PI 55,42% – 59,68%), o SAD B19 - 48,13% (PI 44,68% –51,58%). Mūsų atlikti specifinių vakcininiam RV antikūnų dinamikos tyrimai parodė, kad naudojant SAD Berne vakciną laukinių gūvynų ORV efektyvumo procentas yra aukštesnis, nei naudojant SAD B19

Atliekant tyrimų analizę nustatyta, kad Lietuvoje 2003-2006 metais laukinių ir naminių gyvūnų populiacijose daugėjo pasiutligės teigiamų atvejų dėl netaikomų laukiniams gyvūnams imunoprofilaktikos priemonių. Apibendrinę 2006–2011 m. imunoprofilaktikos nuo pasiutligės rezultatus nustatėme, kad Lietuvos žmonės, o ypač gyvūnai dar nėra saugūs, nors teigiamų pasiutligės atvejų kasmet užregistruojama vis mažiau. Ypač kelia nerimą kasmet registruojami pasiutligės atvejai Baltarusijos pasienyje. Šiuolaikinės Lietuvoje naudojamos pasiutligės vakcinos yra itin efektyvios ir saugios, todėl kilus menkiausiam įtarimui dėl užsikrėtimo pasiutlige būtina pradėti poekspozicinį skiepijimą. Mes galime teigti, kad atliekama laukinių gyvūnų ORV nuo pasiutligės duoda teigiamas rezultatus kovojant su šia pavojinga gyvūnų užkrečiamąja liga. Tačiau mūsų nuomone galbūt reikėtų dar labiau intensyvinti gyvūnų pasiutligės imunoprofilaktikos priemonių taikymą būtent tuose rajonuose, ypač pasienio rajonuose su Baltarusija, kuriuose šiuo metu vis dar nustatomi pasiutligės židiniai.

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