ORIGINAL ARTICLE

First Report on the Efficiency of Oral Vaccination of Foxes against Rabies in Serbia

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Impacts

- Rabies is still one of the most important zoonotic disease of all mammals, which is taken every year around 60 000 human lives.
- Many European countries are rabies free, and for these countries, main threat represents import of rabid animals into the country. In other, infected countries, foxes or other wildlife represent main threat as reservoirs of rabies and are important for transmission of the disease.
- Oral rabies vaccination (ORV) of foxes is the most effective way to control and eradicate rabies in wild animals. In 2010 was launched the programme of ORV of foxes and other wild carnivores in Serbia, and since then, the incidence of rabies has been significantly decreased.

Keywords:
Rabies; elimination; oral vaccination; monitoring; fox

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Summary

Rabies is one of the oldest known zoonotic diseases that has significant impact on public health, but still remains neglected in Serbia. Rabies virus can infect humans and other mammals and causes inflammation of the brain associated with encephalomyelitis and neurological symptoms. In 2010, Veterinary Directorate (national Competent Authority for animal health in Serbia) has started multi-annual project of oral rabies vaccination of foxes and other wild carnivores (e.g. jackals), as support of long-term programme of eradication of rabies in Serbia, co-funded by EU (*financed by Instrument for Pre-Accession Assistance*). Monitoring of the effectiveness of oral vaccination campaigns has been carried out in continuation from 2011 and was based on: (i) *post-mortem* laboratory examination of brain tissue of target animals (foxes, jackals and other carnivores) by fluorescent antibody test (FAT), (ii) detection of antibodies against rabies virus in serum samples by ELISA and (iii) detection of tetracycline biomarker in the mandibles for the evaluation of vaccine bait uptake. From September 2011 to May 2014, the total number of 4943 brain tissue samples, 4241 sera and 4971 mandibles were analysed. Confirmed rabies-positive brains decreased from 10 in 2011/2012 to 6 in 2012/2013 and eventually to 1 positive case in 2013/2014. The seroconversion rate increased from 10.48% (133/1269) in 2011/2012 to 20.11% (362/1800) in 2012/2013 and 42.23% (495/1172) in 2013/2014. Along with the seroconversion, the number of detected tetracycline-positive mandibles demonstrated an increasing tendency in the same period, being 49.67% (682/1373) in 2011/2012, 62.60% (1294/2067) in 2012/2013 and 90.33% (1383/1531) in the monitoring programme carried out in 2013/2014. Presented results confirmed that ORV of foxes and other wildlife in Serbia against rabies was successful and characterized by steady increase of vaccine baits uptake and immunization of animals.

Introduction

Rabies is a neurotropic disease with clinical signs of encephalomyelitis and almost 100% fatality rate. The disease is one of the oldest known zoonosis and is present worldwide, with the exception of Antarctic. All mammals are susceptible to the infection. Almost 60 000 human deaths caused by rabies are reported every year, and most of the victims are children under 15 years (WHO, 2013; Fooks et al., 2014).

The causative agent of rabies is negative-strained RNA virus that belongs to the genus *Lyssavirus*, family *Rhabdovirdae*, order *Mononegavirales*. Genus *Lyssavirus* consist of 12 known and two still undefined species. According to antigenic differences, lyssavirus genus is divided into two main phylogenic groups (I and II), and a novel phylogroup III, that has been recently established. Classical rabies virus (RABV) belongs to phylogroup I (CFSPH, 2012; ICTV, 2013).

Natural reservoirs of classical rabies are different carnivores and bats, while other lyssaviruses are detected only in bats. It is interesting to underline that bats are responsible for classical rabies in North and Latin America, while other lyssaviruses are restricted to bat species in Africa, Asia and Europe (Fooks et al., 2014). In the last few years, many new lyssaviruses were detected in bats: Shimony bat virus in Kenya, Bokeloh bat virus in Germany or Lleida bat lyssavirus in Spain (Kuzmin et al., 2010; Freuling et al., 2011; Ceballos et al., 2013). It seems that all lyssaviruses can infect humans, although there are rare reports of neurological disorders and encephalitis in humans caused by non-RABV lyssaviruses (Johnson et al., 2010a).

Rabies is usually transmitted by a bite of rabid animal, as the virus is present in the saliva. Also, other ways of transmission were reported, especially through transplantation of cornea or solid organs from a donor infected with rabies (Maier et al., 2010; Wallace et al., 2014).

Two epidemiological forms of rabies have been described so far: the sylvatic (wildlife) and urban one. Dogs are the main source of urban rabies in developing countries of Asia, Africa and Latin America, which are characterized by high density of stray dog population and, at the same time, with inadequate vaccination programmes. In the USA and Europe, the mandatory vaccination of dogs and restrictive control of transport and pets moving have led to the elimination of dog-mediated rabies, except in Turkey (Johnson et al., 2010b). In Europe, rabies is maintained in nature in many animal species from family *Canidae*: red foxes and jackals in Central and South part of Europe, wolves and raccoon dogs in northern Europe. Raccoons, coyotes and bats are reservoirs for rabies in America (CFSPH, 2012; Freuling et al., 2013).

Rabies spread in red foxes from Russia to Poland in 1940s. Since then, virus was introduced into majority of European countries and reached France in 1968 (Pastoret and Brochier, 1998; Vitasek, 2004). It seems that rabies virus adapted to the foxes, which represent the principal reservoir of rabies among wildlife in Europe (Pastoret and Brochier, 1998).

After the World War II, rabies existed in former Yugoslavia in both urban and sylvatic form. Extensive veterinary measures and massive vaccination of dogs decreased the number of infected dogs and human exposure. Implementation of these measures resulted in progressive disappearance of urban rabies. The last report of human rabies case was recorded in 1980 (Aylan et al., 2011; Aikimbayev et al., 2014).

In 1977, rabies entered Serbia from the north, probably from Hungary, during the large enzootic emergence in red foxes in Europe. Phylogenetic analysis of Serbian isolates from 1986 to 2002 revealed the possibility of further spreading of rabies over Danube River to Bulgaria and further to the south (McElhinney et al., 2011). Nowadays, the main reservoirs of sylvatic rabies are red foxes. Sometimes, domestic animals are attacked by rabid foxes and rabies can be introduced into population of domestic animals (Petrovic, 1987; Sinkovic, 2008; Maksimovic Zoric et al., 2013).

The first idea of oral vaccination of foxes appeared in 1971, when Baer et al. (1971) succeeded in their experiment to vaccinate red foxes with the attenuated rabies virus. The first country that implemented ORV of foxes was Switzerland in 1978 (Steck et al., 1982). Thereafter, many European countries followed this trial and started the ORV campaigns. During the 1990s, most of the Central and Eastern European countries joined the programme (Vitasek, 2004; Freuling et al., 2013). Meanwhile, some countries that obtained the 'rabies-free' status have announced the re-emergency of the disease - Italy in 2010 and Greece in 2012 and 2013 (Capello et al., 2010; Tsiodras et al., 2013).

In Serbia, according to relevant legislative *(the Law on veterinary matters, Official Gazette of Republic of Serbia No 91/05, 30/10, 93/12, Rulebook on early detection, diagnosis, control and eradication of rabies, Official Gazette RS 78/09, 2009 and the Rulebook on Programme of Animal Health Protection Measures, issued annually)*, vaccination of all cats and dogs is mandatory and rabies is a notifiable disease. All suspect animal cases with neurological disorders and symptoms related to rabies shall be officially notified to Competent Authority (CA), official investigation to be implemented and samples should be submitted to authorized veterinary institute for further laboratory analysis. Brain tissue samples are tested using FAT (Fluorescent antibody test). Also, post-exposure prophylaxis (PEP) for human victims bit by a rabid or rabies-suspect animal is available and free of charge. However, surveillance measures were not sufficient to achieve successful rabies elimination in wildlife animals (Rupprecht et al., 2004; Sinkovic, 2008).

In 2000, a pilot project of oral vaccination of foxes was conducted in one limited area of Serbia by manual distribution of baits. The next trial was conducted in 2002, when the aerial distribution of vaccine baits was performed by parachutes, covering the territory of 500 km^2 in the North Vojvodina. During 18 months of ORV project, no rabies cases were registered in this area (Sinkovic, 2008). In 2010, the European Commission started to support regional veterinary services in West Balkans with the projects of eradication of rabies with long-term implementation of Oral Rabies Vaccination (ORV) of foxes and other wild carnivores (e.g. jackals) funded by IPA projects (*Instrument for Pre-Accession Assistance*).

The aim of this study was to describe the first results of ORV programme for rabies elimination in red foxes and other wild animals in Serbia from 2010 to 2014. The efficacy of rabies elimination will be presented through the assessment of baits uptake, tetracycline depositing in mandibles and seroconversion in immunized animals. Furthermore, the data on animal rabies cases for the period from 2006 to 2014 are presented in this article.

Materials and Methods

Vaccination programme

Oral vaccination of foxes started in November 2010, according to national strategy for rabies eradication (*Strategic operational multi-annual action plan for eradication, control and monitoring of Rabies in Serbia, Veterinary Directorate, October 2010*), with a standard programme of distribution of vaccines twice a year – in autumn and spring. Distribution of baits was carried out from the air by planes, covering the whole territory of the country, except cities, towns, settlements, rivers, lakes and motorways. In total, eight campaigns were completed from the end of 2010, and the 9th campaign started in autumn 2014. During the first four campaigns, 1 400 000 vaccine doses were distributed. Afterwards, the number of distributed baits increased to 1 610 000. The density of baits was 20 baits/km^2 and 23 baits/km^2 in 2011/2012 and 2013/2014, respectively. The distance between two flight lines was approximately 500–600 m, and the distribution has been continuously monitored using a navigation programme. It is planned that 4 foxes/100 km^2 should be submitted each year for the examination, in line with WHO recommendations.

In the first two seasons was used commercial vaccine *Lysvulpen* (Bioveta, Czech Republic), which consists of the attenuated rabies strain SAD Bern (infectious dose 1.8×10^6 $TCID_{50}$–1.8×10^8 $TCID_{50}$). Each of the baits contained 150 mg of biomarker Tetracycline, an indicator of baits uptake assessment. Starting from the seventh campaign, in October 2013, vaccination was conducted with *Fuchsoral* vaccine, containing strain SAD B19 (IDT Biologica, Germany).

Laboratory testing

From 2006 to 2011, laboratory testing for rabies surveillance of indicator animals was performed in two institutions: 'Pasteur Institute' in Novi Sad and 'Scientific Veterinary Institute of Serbia' in Belgrade. From autumn 2011, after two campaigns of vaccine distribution, monitoring programme of vaccination efficacy was implemented. Three veterinary institutes were supplied with up to date equipment and trained laboratory experts for laboratory diagnostic of rabies post-vaccination monitoring programme – 'Institute of Veterinary Medicine of Serbia' in Belgrade (NIVS), Scientific Veterinary Institute 'Novi Sad' in Novi Sad (NIV-NS) and Veterinary Institute 'Kraljevo' in Kraljevo (VSI Kraljevo). Other regional institutes and hunter associations were responsible for collecting and submission of carcasses and samples of foxes and other wildlife carnivores. After opening the cadavers, brain samples, blood and blood exudates and mandibles are taken for laboratory analysis.

Brain samples

As there are no pathognomonic gross findings of rabies infection *post-mortem*, the most reliable diagnosis is detection of virus in brain samples after necropsy. Identification of viral antigen was performed using fluorescent antibody test (FAT), which is recommended laboratory technique by the OIE (OIE Terrestrial Manual, 2013). Brain samples were taken after opening the fox skull. For a successful analysis is important to collect fresh samples of thalamus, medulla oblongata and Ammon's horn. Impressions from brain tissue were made on microscope slides and fixed by drawing through the flame. Detection of the antigen was performed using commercial conjugate containing anti-rabies IgG antibodies conjugated with fluorescein isothiocyanate FITC, produced by Bio-Rad, France (*AntiRabies Nucleocapsid Conjugate*) or by Sifin, Germany (*Monoclonal Anti-Rabies, FITC*). Brain samples were examined with the fluorescence microscope (Axio Observer A1, Carl Zeiss, Gottingen, Germany). In positive samples, the presence of typical yellow-green coloured inclusions represent agglomerated nucleocapsid proteins in neurons, whereas no fluorescence is present in negative samples. Fluorescent antibody test enables detection of rabies virus with a confidence level 95–98% and therefore is the most used and reliable method.

Since the beginning of monitoring of ORV in 2011, all FAT positive or doubtful rabies cases were analysed byRT-PCR at NIVS, Belgrade, according to *Diagnostic manual for control of oral vaccination of foxes and other wildlife against rabies* (Veterinary Directorate, 2010). RT-PCR detects RNA sequences from the genome section of the rabies virus encoding the N- and P-protein. The primers used are the part of genome sequence of SAD B19 vaccine virus strain and specific for genotype I (Rabies virus) (East et al., 2001). Confirmed, positive cases are being differentiated between field and vaccine strain of rabies on the territory of ORV using RFLP analysis (Restriction Fragment Length Polymorphism). Enzyme Tsp45I (NmuCI (Tsp45I) (10 U/μL), Thermo Scientific) specifically splices vaccine strain (SAD B19-derivate) at position 182 of the NP-fragment. Two separated fragments on electrophoresis indicate the presence of vaccine viral strain and intact fragment of 367 bp confirms the presence of field strain.

In the case of human exposure or injury, animals' brain samples are submitted to 'Pasteur Institute' in Novi Sad for diagnostic examination by FAT and virus isolation on cell culture.

Serological examination

Serological screening is important to measure seroconversion rate in fox serum samples during the vaccination campaigns, after the consumption of baits. Anti-rabies IgG antibodies were detected by indirect enzyme-linked immunosorbent assay (ELISA). During 2011/2012 and 2012/2013 campaigns, Platelia Rabies II ELISA kit (Bio-Rad, Paris, France) was used, according to manufacturer's instructions. The test is suitable both for detection of antibodies (qualitative method) and determination of antibody levels (quantitative method). At least, 0.5 EU/ml was accepted as a minimum for the positive serum to exclude false-positive results. This test has been previously validated and described elsewhere (Servat et al., 2007). In the season 2013/2014, samples were tested with BioPro Rabies ELISA Ab Kit (BioPro, Prague, Czech Republic), validated by Wasniewski and Cliquet (2012).

Detection of tetracycline

Biomarker tetracycline provides data of baits uptake with the characteristic to deposit in bones. For this purpose, the lower jaw was cut at a thickness of 0.2 to 0.6 mm with a low-speed saw (IsoMet, Buehler). Tetracycline fluoresces under the ultraviolet microscope light and typical yellow lines appear around veins in mandibles and in canine dentine and cementum (Johnston et al., 1999). The number of tetracycline lines depends on the number of consumed baits and the age of the animal. The age of the animal is determined by the diameter of dental pulp, the presence/absence of tooth cementum and the number of dark lines in cementum, which corresponds to the number of winters that fox survived. The age of the animal is expressed as: cub (under 12 months) or adult (over 12 months) (Veterinary Directorate, 2010).

Statistics

The obtained data were analysed using LABIS computer program.

Results

Rabies epizootic situation from 2006 to 2014

Assessment of rabies epizootic situation between 2006 and 2014 is presented in Tables 1 and 2. During surveillance, from 2006 to 2014, 3816 brain samples were tested by FAT and 922 (24.16%) reacted positive (Table 1). Within monitoring of ORV effectiveness, rabies was detected in 17 (0.34%) of 4943 brain samples analysed by FAT (Table 2). From 2010, the number of rabid animals was reduced dramatically, which corresponds to the beginning of ORV of foxes in Serbia. The number of rabies cases varied from 233 in 2008 to 1 in 2014. From 2006 to 2009, positive cases represented almost one-third of all examined samples (average 31.86%). From 2012, the total number of 26 FAT-positive

Table 1. Surveillance of rabies in Serbia between 2006 and 2014

Year	No. of tested animals	No. of positive cases	% positive cases
2006	549	192	34.97%
2007	528	160	30.30%
2008	740	233	31.49%
2009	590	181	30.68%
2010	462	104	22.51%
2011	409	43	10.51%
2012	271	9	3.32%
2013	167	0	0.00%
2014	100	0	0.00%
Total	**3816**	**922**	**24.16%**

Table 2. Monitoring of ORV of foxes and other wild animals in Serbia between 2012 and 2014

Year	No. of tested animals	No. of positive cases	% positive cases
2012	1370	10	0.72%
2013	2069	6	0.29%
2014	1504	1	0.07%
Total	**4943**	**17**	**0.34%**

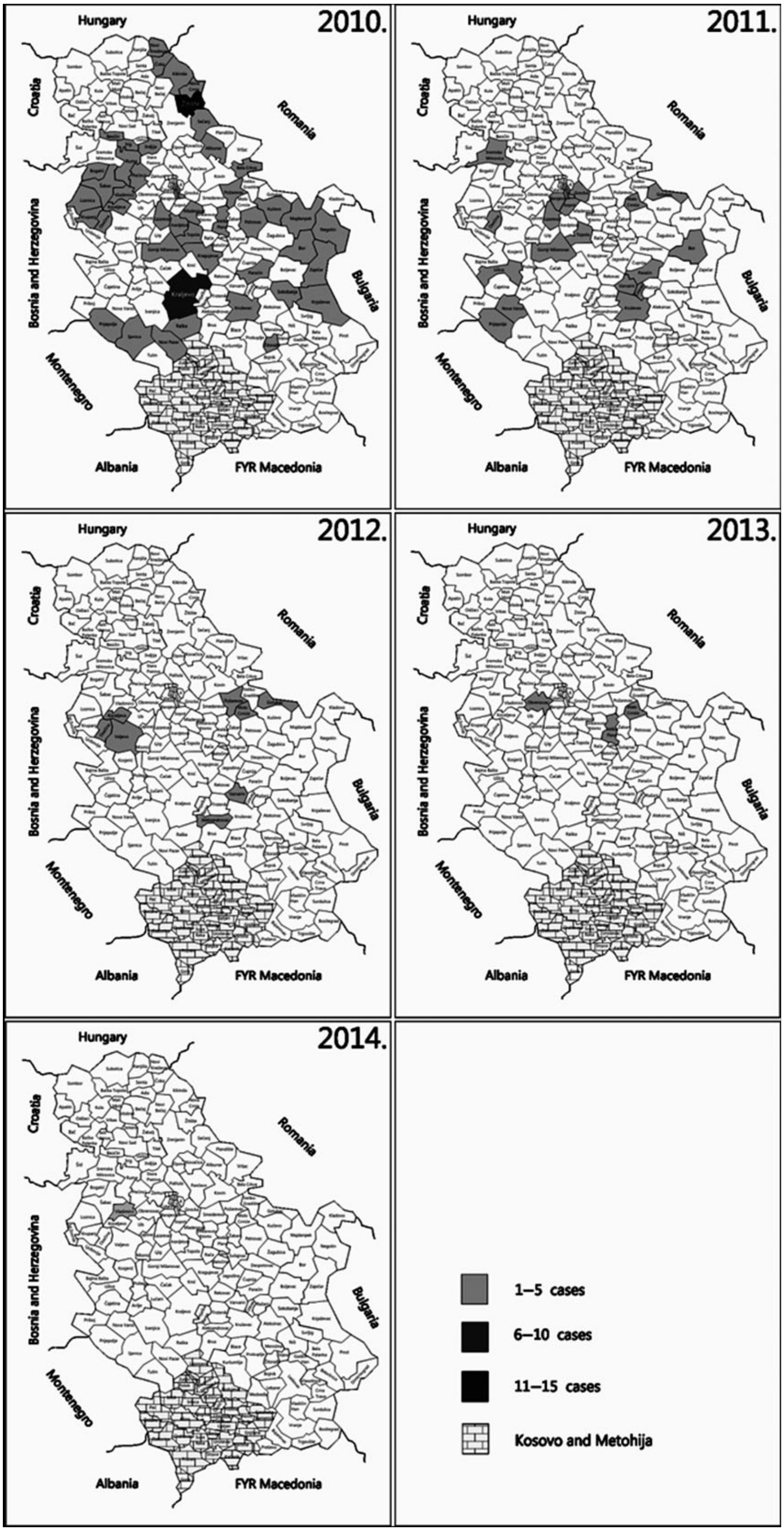


Fig. 1. Prevalence of rabies in Serbia from 2010 to 2014; Data source: Pasteur Institute, Novi Sad, Serbia (www.paster.org.rs).

brain samples were examined by RT-PCR and all tested cases belonged to RABV field strain.

In 2010, before implementation of ORV of foxes, rabies was widespread distributed all over the country (Fig. 1). It is evident that most of rabies cases were registered near the border lines to Romania, Bosnia and Herzegovina, Montenegro and Bulgaria. In the central part of Serbia, the majority of rabid animals originated from Municipality of Kraljevo. Sudden outbreak of rabies occurred in 2011, when 17 foxes out of 43 positive animals were detected in Belgrade and its surrounding. In 2012, rabies was still present in the municipalities near Romania and Bosnia and Herzegovina; however, a significant decrease in the number of rabies cases and positive effects of oral vaccination with only 19 confirmed cases has been observed. In 2013 were detected five foxes and one jackal, mostly in the region of Belgrade city. In 2014 was only one registered positive fox in Vladimirci municipality.

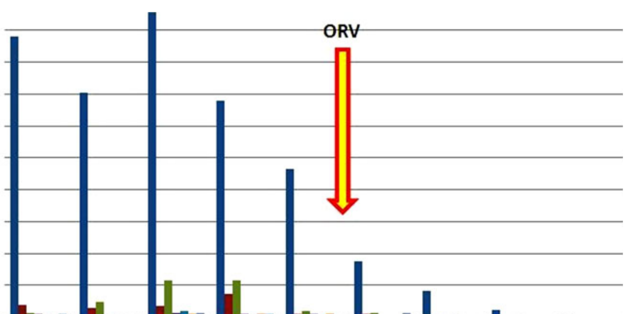
Analysed by animal species, the highest prevalence of rabies was recorded in the population of foxes and just sporadically in other wildlife or in domestic animals (Fig. 2). Foxes made 84.66% (795/939) of all positive samples. The proportion of foxes in the total number of positive cases per year from 2006 to 2014 was 176 (91.67%), 141 (88.13%), 191 (81.97%), 136 (75.14%), 93 (89.42%), 35 (81.39%), 17 (89.47%), five (83.33%) and one (100%), respectively. The incidence of infection in wildlife and domestic animals was 87.33% (820/939) and 12.67% (119/939), respectively. The occurrence of rabies in wildlife animals was 6.9-fold higher than in domestic animals (820 versus 119). Regarding rabies cases for the observed period of 8 years (2006–2014), in the population of domestic animals, the disease was more frequently recorded in cats rather than dogs (7.13% versus 4.26%). After the first campaign of oral vaccination of foxes in autumn 2010, the number of positive rabies cases decreased steadily from 43 in 2011 to 19 in 2012, six in 2013 and finally to only one registered fox in January 2014.

Monitoring of oral vaccination of foxes and other wild carnivores

Results of the monitoring programme of oral vaccination of foxes and other carnivores are presented in Table 3. The samples were collected from September of current year to May next year. From the beginning of oral vaccination in autumn 2010, a total of 4943 brain samples, 4241 sera and 4971 mandibles were tested. During 2011/2012, the number of examined brain samples was 1370, followed by 1269 sera and 1373 mandibles. During the next hunting season in 2012/2013, 2069 brain samples, 1800 sera and 2067 mandibles were analysed. In 2013/2014, the number of tested samples decreased a bit to 1504 brain samples, 1172 sera and 1531 mandibles. Approximately, about 85–88% of all shot animals were foxes, 11–13% jackals and 1–2% belonged to the other species such as wolves, wild cats and mustelines (data not presented).

In 2011/2012, 10 (0.73%) tissue brain samples resulted positive in FAT. In 2012/2013, six (0.29%) rabies-positive brains were detected, whereas only one (0.07%) case was confirmed in the season 2013/2014. All rabies cases belonged to rabies field strain.

Along with the decrease in number of confirmed rabid animals, seroconversion rate increased from 10.48% (133/1269) in 2011/2012 to 20.11% (362/1800) in 2012/201 and 42.23% (495/1172) in 2013/2014. Moreover, the



	2006	2007	2008	2009	2010	2011	2012	2013	2014
Fox	176	141	191	136	93	35	17	5	1
Dog	8	6	7	15	2	2			
Cat	3	10	23	23	4	3	1		
Jackals	2	1	3	2	2		1	1	
Wild cat	1	1	4	1					
Other wildlife animals			2	2	2				
Other domestic animals	2	1	3	2	1	3			

Fig. 2. Prevalence of rabies in different animal species between 2006 and 2014.

examination of tetracycline accumulation in the lower jaws revealed continuous increase in the number of consumed baits over the years. Biomarker positive mandibles ranged from 49.67% (682/1373) to 62.60% (1294/2067) and 90.33% (1383/1531) for the same observed three-season period.

Results of the oral vaccination according to the age of the animals are presented in Table 4. The number of analysed adult animals was significantly higher compared to the number of cubs younger than 12 months (3814 versus 1129, 77.16% versus 22.84%). It is also evident that anti-rabies IgG antibodies were more often detected in adult animals then in cubs (782 versus 208, 78.99% versus 21.01%). Tetracycline was also more frequently detected in adult animals compared to cubs (2616 versus 743, 77.88% versus 22.12%).

The territory of Serbia is administratively divided into 25 districts (24 districts and city of Belgrade as a separate district). The geographic overview of tested and positive samples analysed by FAT, ELISA and TC labelling between 2011 and 2014 is shown in Tables 5, 6 and 7. In general, the deposition of tetracycline in mandibles is higher than the seroconversion rate. As a consequence of poor immunization, some rabies-positive cases were registered in the areas where no one animal seroconverted, although bait uptake was satisfactory (Branicevski district in 2011/2012 and 2013/2014 and Podunavski district in 2012/2013 (in bold).

Table 3. Results of FAT, ELISA and TC biomarker in foxes and other wild animals after ORV in Serbia from 2011 to 2014

		Diagnostic examination					
		FAT (Ag)*		ELISA (At)†		TC‡	
Year	Institution	Positive	Negative	Positive	Negative	Positive	Negative
2011–2012	NIVS	9	616	57	503	367	261
	VSI Kraljevo	1	372	38	335	130	243
	NIV-NS	0	372	38	298	185	187
		10 (0.73%)	1360 (99.27%)	133 (10.48%)	1136 (89.52%)	682 (49.67%)	691 (50.33%)
Total		1370		1269		1373	
2012–2013	NIVS	6	774	108	495	542	236
	VSI Kraljevo	0	746	153	593	454	292
	NIV-NS	0	543	101	350	298	245
		6 (0.29%)	2063 (99.71%)	362 (20.11%)	1438 (79.89%)	1294 (62.60%)	773 (37.40%)
Total		2069		1800		2067	
2013–2014	NIVS	1	679	231	202	635	48
	VSI Kraljevo	0	437	150	287	393	68
	NIV-NS	0	387	114	188	355	32
		1 (0.07%)	1503 (99.93%)	495 (42.23%)	677 (57.77%)	1383 (90.33%)	148 (9.67%)
Total		1504		1172		1531	
(2011–2014) Total		4943		4241		4971	

*FAT (Ag) – rabies antigen detected by FAT.
†ELISA (At) – anti-rabies IgG antibodies detected by ELISA.
‡TC – tetracycline biomarker.

Table 4. Age of foxes and other wild carnivores in relation to seroconversion and bait uptake

	<12 months			>12 months		
Year	No. of tested animals/total no.	ELISA positive/total positive	TC positive/total positive	No. of tested animals/total no.	ELISA positive/total positive	TC positive/total positive
2011/2012	268 (23.74%)	17 (8.17%)	106 (14.27%)	1099 (28.81%)	116 (14.83%)	576 (22.19%)
2012/2013	502 (44.42%)	68 (32.69%)	319 (42.93%)	1567 (41.08%)	294 (37.59%)	975 (37.27%)
2013/2014	359 (31.77%)	123 (59.13%)	318 (42.80%)	1148 (30.10%)	372 (47.57%)	1065 (40.71%)
Total	**1129 (22.84%)**	**208 (21.01%)**	**743 (22.12%)**	**3814 (77.16%)**	**782 (78.99%)**	**2616 (77.88%)**

Table 5. Geographic distribution according to results of FAT, ELISA and TC detection in 2011/2012

District	FAT (%) positive/total positive	ELISA (%) positive/total positive	TC (%) positive/total positive
Borski	0 (0.00%)	9 (6.77%)	41 (6.01%)
Braničevski	**3 (30.00%)**	**0 (0.00%)**	**33 (4.84%)**
City of Belgrade	0 (0.00%)	9 (6.77%)	38 (5.57%)
Jablanički	0 (0.00%)	2 (1.50%)	9 (1.32%)
Južno-bački	0 (0.00%)	5 (3.76%)	35 (5.13%)
Južno-banatski	1 (10.00%)	13 (9.77%)	65 (9.53%)
Kolubarski	3 (30.00%)	7 (5.26%)	47 (6.89%)
Mačvanski	1 (10.00%)	11 (8.27%)	55 (8.065%)
Moravički	0 (0.00%)	4 (3.01%)	9 (1.32%)
Nišavski	1 (10.00%)	3 (2.26%)	18 (2.64%)
Pčinjski	0 (0.00%)	0 (0.00%)	9 (1.32%)
Pirotski	0 (0.00%)	2 (1.50%)	6 (0.88%)
Podunavski	0 (0.00%)	0 (0.00%)	6 (0.88%)
Pomoravski	0 (0.00%)	1 (0.75%)	19 (2.79%)
Rasinski	0 (0.00%)	0 (0.00%)	0 (0.00%)
Raški	0 (0.00%)	5 (3.76%)	11 (1.61%)
Severno-bački	0 (0.00%)	5 (3.76%)	18 (2.64%)
Severno-banatski	0 (0.00%)	8 (6.02%)	29 (4.25%)
Srednje-banatski	0 (0.00%)	10 (7.52%)	44 (6.45%)
Sremski	0 (0.00%)	3 (2.26%)	31 (4.55%)
Šumadijski	1 (10.00%)	2 (1.50%)	27 (3.96%)
Toplički	0 (0.00%)	2 (1.50%)	5 (0.73%)
Zaječarski	0 (0.00%)	5 (3.76%)	36 (5.28%)
Zapadno-bački	0 (0.00%)	7 (5.26%)	28 (4.11%)
Zlatiborski	0 (0.00%)	20 (15.04%)	63 (9.24%)
Borski	**10**	**133**	**682**

Table 6. Geographic distribution according to results of FAT, ELISA and TC detection in 2012/2013

District	FAT (%) positive/total positive	ELISA (%) positive/total positive	TC (%) positive/total positive
Borski	0 (0.00%)	16 (4.42%)	62 (4.79%)
Braničevski	0 (0.00%)	0 (0.00%)	89 (6.88%)
City of Belgrade	1 (16.66%)	9 (2.49%)	70 (5.41%)
Jablanički	0 (0.00%)	10 (2.76%)	34 (2.63%)
Južno-bački	0 (0.00%)	21 (5.80%)	65 (5.02%)
Južno-banatski	0 (0.00%)	31 (8.56%)	112 (8.66%)
Kolubarski	4 (66.66%)	3 (0.83%)	32 (2.47%)
Mačvanski	0 (0.00%)	21 (5.80%)	68 (5.26%)
Moravički	0 (0.00%)	33 (9.12%)	69 (5.33%)
Nišavski	0 (0.00%)	18 (4.97%)	76 (5.87%)
Pčinjski	0 (0.00%)	6 (1.66%)	27 (2.09%)
Pirotski	0 (0.00%)	1 (0.28%)	14 (1.08%)
Podunavski	**1 (16.66%)**	**0 (0.00%)**	**25 (1.93%)**
Pomoravski	0 (0.00%)	0 (0.00%)	4 (0.31%)
Rasinski	0 (0.00%)	12 (3.31%)	34 (2.63%)
Raški	0 (0.00%)	14 (3.87%)	36 (2.78%)
Severno-bački	0 (0.00%)	23 (6.35%)	43 (3.32%)
Severno-banatski	0 (0.00%)	11 (3.04%)	30 (2.32%)
Srednje-banatski	0 (0.00%)	10 (2.76%)	48 (3.71%)
Sremski	0 (0.00%)	23 (6.35%)	78 (6.03%)
Šumadijski	ND	ND	ND
Toplički	0 (0.00%)	1 (0.28%)	7 (0.54%)
Zaječarski	0 (0.00%)	28 (7.73%)	80 (6.18%)
Zapadno-bački	0 (0.00%)	13 (3.59%)	34 (2.63%)
Zlatiborski	0 (0.00%)	58 (16.02%)	157 (12.13%)
Total	**6**	**362**	**1294**

ND, not done.

Table 7. Geographic distribution according to results of FAT, ELISA and TC detection in 2013/2014

District	FAT (%) positive/total positive	ELISA (%) positive/total positive	TC (%) positive/total positive
Borski	0 (0.00%)	10 (2.02%)	43 (3.11%)
Borski	0 (0.00%)	10 (2.02%)	43 (3.11%)
Braničevski	**1 (100.00%)**	**0 (0.00%)**	**108 (7.81%)**
Braničevski	0 (0.00%)	0 (0.00%)	108 (7.81%)
City of Belgrade	0 (0.00%)	20 (4.04%)	40 (2.89%)
Južno-bački	0 (0.00%)	24 (4.85%)	87 (6.29%)
Južno-banatski	0 (0.00%)	69 (13.94%)	99 (7.16%)
Kolubarski	0 (0.00%)	49 (9.90%)	74 (5.35%)
Mačvanski	0 (0.00%)	44 (8.89%)	83 (6.00%)
Moravički	0 (0.00%)	22 (4.44%)	91 (6.58%)
Nišavski	0 (0.00%)	28 (5.66%)	69 (4.99%)
Pčinjski	0 (0.00%)	0 (0.00%)	0 (0.00%)
Pirotski	0 (0.00%)	3 (0.61%)	8 (0.58%)
Podunavski	0 (0.00%)	0 (0.00%)	28 (2.02%)
Pomoravski	0 (0.00%)	4 (0.81%)	43 (3.11%)
Rasinski	0 (0.00%)	11 (2.22%)	26 (1.88%)
Raški	0 (0.00%)	21 (4.24%)	65 (4.70%)
Severno-bački	0 (0.00%)	17 (3.43%)	35 (2.53%)
Srednje-banatski	0 (0.00%)	24 (4.85%)	68 (4.92%)
Sremski	0 (0.00%)	32 (6.46%)	104 (7.52%)
Šumadiski	0 (0.00%)	11 (2.22%)	75 (5.42%)
Zaječarski	0 (0.00%)	24 (4.85%)	42 (3.04%)
Zapadno-bački	0 (0.00%)	17 (3.43%)	61 (4.41%)
Zlatiborski	0 (0.00%)	65 (13.13%)	134 (9.69%)
Total	**1**	**495**	**1383**

Discussion

The population of red foxes is the main reservoir of rabies in Serbia (Aikimbayev et al., 2014). Many publications reported red foxes as the most important vector for the maintenance of rabies in wildlife in Central and South Europe (Pastoret and Brochier, 1998; Bourhy et al., 1999; Finnegan et al., 2002; Wandeler, 2004).

From 2006 to 2014, 939 rabies cases (922 in surveillance and 17 in monitoring of ORV) were confirmed by FAT and the majority of positive cases, 84.66%, were diagnosed in foxes. Sporadically, rabies has been detected in jackals, wolves and wild cats. Domestic animals, mostly cats and dogs, account for 12.67% of all rabies incidences. In Slovenia and Croatia, more than 90% of all positive rabies cases are diagnosed in red foxes, while dogs and cats make only 4–8% of infected animals (Hostnik et al., 2006; European Commission, 2012a). In Montenegro, rabies is also dominant in red foxes, which account for 80% of all positive rabid wild animals (Pötzsch et al., 2012).

Epizootic situation in Serbia depends mainly on the situation in neighbouring countries. The majority of rabid foxes in Serbia are detected near borders to Romania, Bulgaria and Bosnia and Herzegovina, where rabies has endemic characteristics (Najar and Streinu-Cercel, 2012; Ilieva, 2013; Tošić et al., 2013). The highest risk of disease spreading was reported in 2008, when 233 rabies cases were identified. At the same time, many countries in Balkan region announced elevated number of rabies incidences: 1061 in Croatia, 1089 in Romania, 83 in Bosnia and Herzegovina, 54 in Bulgaria and 43 in Montenegro, and also 3353 cases were reported in Russian Federation and 2164 in Ukraine (WHO Rabies-Bulletin-Europe, 2008). A stable epizootic situation without any detected rabies-positive fox has been present for couple of years in the northern part of Vojvodina that borders Hungary. In 1992, Hungary started an ORV programme of foxes and since then significantly reduced sylvatic rabies (Vitasek, 2004; Freuling et al., 2013). Epizootic situation in the southern regions, near the borderline to Macedonia, is also considered satisfactory. Macedonia did not report any rabies incidence until 2011, when the first case was diagnosed (Kirandjiski et al., 2012). It is interesting to draw attention to the increased number of positive rabies foxes recorded in the city of Belgrade in 2011, when rabies was confirmed in 17 foxes. Such an emergency of rabies corresponds with the poor distribution of vaccine baits near towns during the first three campaigns, from autumn 2010 to the end of 2011.

Sporadically, rabies can spill over from wildlife to domestic animals, which can further attack humans. In 2011, six attacks by rabid cats and dogs were registered, whereas one attack was reported in 2012. There were no human attacks by rabid animals in 2013 and 2014 (Pasteur Institute - Novi Sad, Serbia, 2014). Rabid cats were predominant domestic species responsible for virus transmission compared to dogs (7.13% versus 4.26%). This could be attributed to the lack of mandatory vaccination of cats, their rapid reproduction and a huge number of stray cats. With the initiation of ORV of foxes in 2010 in Serbia, the number of positive rabies cases in domestic animals decreased rapidly. Cases of humans attacked by rabid cats and dogs were also reported in Romania and Bulgaria, but incidence of human attacks was reduced after implementation of ORV of foxes (Najar and Streinu-Cercel, 2012; Ilieva, 2013).

Oral vaccination of foxes is proven, effective method for the elimination of rabies in wildlife population. In Europe, 24 countries have already implemented ORV of foxes (Freuling et al., 2013). The evaluation of monitoring of ORV was reported for Germany, Poland, Estonia, Slovenia, Czech Republic and other countries (Smreczak and Zmudzinski, 2005; Hostnik et al., 2006; Matouch et al., 2006; Cliquet et al., 2012; Müller et al., 2012). The first campaign was carried out in Switzerland in 1978, and until 1994 rabies was reduced in Europe to <20% of the initial number of cases (Vitasek, 2004). In 1978 in Europe were registered 17.202 rabid animals and this number decreased to 7.581 in 2010 (Freuling et al., 2013).

Oral vaccination of foxes is considered successful if the number of positive rabies cases is reduced for more than 90% in the period up to 10 years, but in many countries, this aim was achieved during the period of 5 years (Freuling et al., 2013). Several countries in Balkan region started the programme of oral vaccination: Bulgaria in 2009, Kosovo in 2010, Romania in 2011, Croatia in 2011, Macedonia in 2011, Montenegro in 2011, and Bosnia and Herzegovina in 2011. All these countries reported significant reduction of rabies incidence after the implementation of the programme. For example, Bulgaria declared 47 positive foxes in 2009 and one positive case in 2011 (Cvetkovikj et al., 2012; European Commission, 2012a,b; Pötzsch et al., 2012; Ilieva, 2013; Tošić et al., 2013; Yakobson et al., 2014).

Evaluation of the efficiency of oral vaccination in Serbia was monitored by detection of virus antigen in brain samples, seroconversion rate in sera and the presence of biomarker in mandibles. The total number of examined samples included 4943 brains, 4241 sera and 4971 mandibles. During the monitoring of ORV campaigns, 10 rabid animals were registered in 2011/2012, six in 2012/2013 and one rabid fox in 2013/2014. At the same time, seroconversion rate raised steadily from 10.48% to 20.11% and 42.23% from 2011 to 2014. The number of tetracycline-positive lower jaws was higher in comparison with immunization rate – 49.67%, 62.60% and 90.33% for the same observed period. Our results are in accordance with the results from Lithuania and Estonia that also reported higher tetracycline accumulation in fox mandibles and lower humoral response (Cliquet et al., 2012; Zienius et al., 2014). This discrepancy between seroconversion rate and bait consumption can have several reasons. Weak post-vaccination immunity is possible to explain by poor vaccine thermostability and quality. The vaccine efficiency can be reduced with temperature fluctuation (especially in late spring or late summer), and also with rainfalls or high temperatures over 30°C. (Cliquet, 2007; European Commission (c), 2002). In such instances tetracycline remains active, while vaccine titre can be lost. Strong winters with low temperatures, hot summers and rainy periods are probably not suitable for vaccines with SAD strain, which were used in Serbia. According to data from the available literature, better results were achieved with SAG2 vaccine, which can resist repeated thawing and freezing and is more stable at higher temperatures (Flamand et al., 1993). The second reason could be the low sensitivity of commercial ELISA. Knoop et al. (2010) reported that sensitivity of commercial immunoassay was only 32.4% compared to standard Rapid Fluorescent Focus Inhibition Test (RFFIT) and concluded that ELISA can give false-negative results. In some occasions, foxes eat the bait without perforation of blister and, consequently, consume tetracycline but not the vaccine (Cliquet et al., 2012; Ilieva, 2013). Finally, it is very important to be careful about calendar and term of baits distribution. Some campaigns in

Serbia were postponed and it was recorded that any delay from planned schedules can spoil successfulness of vaccination (Cliquet and Aubert, 2004).

The results of our investigation showed that the number of tested older foxes and other wild carnivores was higher than the number of young cubs (3814 versus 1129). In parallel, monitoring of seroconversion rate and tetracycline consumption revealed that young animals took less vaccine baits in comparison with adult animals. Immunization rate was 21.01% for cubs and 78.99% for older animals. Tetracycline was also more often detected in adults (77.88%) then in cubs (22.12%). We assumed that such results are probably due to the fact that cubs are too small to consume the bait, especially during early-spring campaigns. Another problem may pose the presence of maternal antibodies, which can prevent the development of active immunity. Bruyere et al. (2000) suggested that spring vaccination should be performed late in May or June instead of April, because young foxes are not strong enough to consume the vaccine. In southern part of Europe, where Serbia is located, the vixens give birth later, thus baits distribution in April is less efficient (Hostnik et al., 2003).

Conclusions

Oral vaccination of foxes had substantial impact on the reduction of the number of positive rabies cases in Serbia. After nine campaigns of ORV, the number of rabies-positive foxes has declined significantly from 93 in 2010 to only one case in 2014. Implementation of ORV of foxes also had a positive effect on other animal species. Among the most susceptible wildlife populations are jackals and wild cats. The last case of rabid wild cat was recorded in 2009 and jackal in 2013. Regarding domestic animals, rabies occurred mainly in cats and dogs, as a consequence of sylvatic rabies. The number of rabid dogs and cats is also significantly reduced. In 2009 were registered 15 rabid dogs and 23 cats. The last rabies-positive dog was confirmed in 2011 and rabid cat in 2012.

Year by year, the baits uptake shows constant increasing tendency and the post-vaccinal immunization rate is higher. The vaccination in Serbia should be continued for at least another two years after recording the last rabies case. Extending the programme of oral vaccination of foxes to other countries is of paramount importance, because only a comprehensive and coordinated action can result in permanent elimination of rabies.

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