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EVALUATION OF THE ORAL RABIES VACCINATION PROGRAM OF RED FOXES (*Vulpes Vulpes*) POPULATION IN ROMANIA IN 2014

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Abstract
Rabies is a fatal zoonotic viral disease produced by a Lyssavirus and is causing more than 70,000 human deaths each year. In Romania foxes are the main wildlife reservoir. Oral rabies (ORV) vaccination of this specie is the most effective method to control and eventually eradicate rabies. Supported by co-financing program between Romania and European Union, successive ORV campaigns were conducted. Monitoring of the effectiveness of oral vaccination campaign has been carried out continuously from 2011 and was based on: (i) post-mortem laboratory examination of brain tissue of foxes by fluorescent antibodytest (FAT), (ii) detection of antibodies against rabies virus in thoracic liquid by ELISA, (iii) detection of tetracycline biomarker in teeth for the evaluation of vaccine bait uptake and (iv) discrimination between wild and vaccinated rabies virus strains by PCR and sequencing techniques. The laboratory analysis of 2014 campaign revealed 31.18% seropositivity and detection of tetracycline biomarker in 55.4% of the foxes tested. No vaccine-induced rabies cases occurred and all positive foxes (1.33%) were infected with wild rabies strains present in Romania.

Key words: *rabies, oral vaccination program, foxes, vaccine, baits.*

INTRODUCTION

Rabies is a Central Nervous System zoonotic disease, with the causative agent Rabies virus, the negative-sense single stranded RNA viruses of the Lyssavirus genus within the family *Rhabdoviridae*, divided into seven genotypes. Genotype 1 viruses is distributed worldwide and generally is found in terrestrial mammals and causes between 37.000 and 87.000 human deaths annually (Virus taxonomy 9th report, 2012; WHOExpert Consultation on Rabies second report, 2013).In Europe canine rabies has been eradicated from developed countries by control measures such as dog movement restriction and mass vaccination and now the major reservoir of rabies was replaced by wild animals, especially red fox (*Vulpes vulpes*) (Cliquet, 2015). Extensive oral vaccination programs (ORV) with baits for red foxes have reduced the incidence of rabies in many Western European countries (Slate, 2009; Zienius, 2011). In Romania dog mediated rabies was predominant before and after World War II with dogs accounting for about 75% of all rabies cases in 1946. When the fox rabies epizootic that spread throughout most of Europe reached Romania, rabies cases in foxes increased over those in dogs (Avram, 20006; Turcitu, 2010).

A co-financed by the EU and the Romanian state budget oral vaccination trial of foxes has been conducted in 16 counties from the western part of Romania in spring and autumn 2011. From 2012 the OV programs were implemented throughout the Romanian territory.

The objective of this study is to assess the effectiveness of the ORV monitoring program in 2014.

MATERIALS AND METHODS

Study area

The vaccination area of this study involved of the entire Romanian territory (237.500 km2). Romania is situated in the East of Europe and it has borders with Moldova to the Northeast, Ukraine to the North and East, Hungary and Serbia to the West and Bulgaria to the South. The Black Sea forms the Eastern border of the country (Turcitu, 2010). According to the cen-

tral authorities statistics the population of foxes for the year 2014 is estimated at 62.707 foxes.

Vaccine

Lysvulpen vaccine (Bioveta®, Czech Republic) has been used for ORV program. According to the vaccine data sheet, it contains SAD Bern modified attenuated strain cells (min 1.8 × 107 TCID50-max1.8 × 108 TCID50/bait). In one bait, there is one vaccination virus dose (1.8 ml) closed in aluminum-plastic blister. Round, dark brown bait is made of feed mixture attractive for foxes and other target animal species. Each bait contains 150 mg of tetracycline HCl, which is intended as an indicator of ingestion by target animal species (Lysvulpen data sheet). Baits were stored in freezers at -20°C prior to use and during the entire vaccination campaign as well.

Baits distribution

For 2014 the co-financing program for surveillance, control and eradication of rabies in Romania approved by Commission Decision 2013/722/CE, submitted by Romania, provides application on the entire territory of Romania, in 41 counties. The bait distribution includes border with Serbia, Hungary, Ukraine, Moldova and Bulgaria. The vaccination has been performed in only one campaign, September-October, because of tendering problems related to the procurement and aerial distribution of vaccine baits. The vaccination of foxes was carried out by air distribution of baits from 8 aircraft (number of 5.325.200 baits with an approx. 25 baits/km2), with a distance between flight lines of 500 meters and 150 meters altitude, by avoiding the territories of localities, water surfaces, highways, etc. Estimated surface suitable for aerial vaccination is approximated at 213.375 square kilometers. It has been done a manual distribution around localities and areas difficult to reach by plane (number of 75.400 of baits, approximately 25 baits/km^2). The data were recorded on Geographical Identification System (GIS) using Geographical Positioning System (GPS).

Samples collection

At a 45 days following vaccination campaign, there shall be performed the hunting of foxes in order to assess the efficiency of vaccination, for this purpose, there shall be shot 4 foxes/year/100 km^2. Brain samples were collected as previously described (EFSA Scientific report, 2010). Field blood samples were generally collected from the shouted foxes thoracic cavity. Samples were stored at -20°C until use for ELISA testing. The foxes lower jaws were collected from each sample.

ORV program monitoring

Taking into account the high incidence of rabies cases in Romania, and the fact that some infected foxes do not show any clinical/nervous symptoms when there are shot, there has been decided that all foxes shall be tested by standard method such as fluorescent antibody test (FAT) used for the detection of rabies virus antigen on brain samples (OIE, 2014). Different commercial immunofluorescent conjugates (Bio-Rad, Fujirebio, Rabitest) were used in order to perform this test. FAT negative samples are sent to NRL for further investigations. The 150 μm sections throughout canine tooth with some alveolar bone tissue (using ISOMET Low Speed Saw) have been performed from each lower jaw. Samples of tooth and surrounding alveolar bone were tested at the NRL for rabies by specific fluorescence to detect tetracycline deposits (excitation filter 380-425 nm, barrier 460 nm). The age of all the foxes tested was determined on the basis of dental examination (cub, younger than 12 months of age, adult older than 12 months (Cliquet, 2012). Immune response was assessed using the indirect enzyme-linked immunosorbent assay *(ELISA)* method (Bio-Rad Platelia Rabies II Kit, France). Assays were done in a 96 wells microplate, coated with rabies virus glycoprotein according to the producer recommendations. 0.5 EU/ml or above antibody titers were expressed as positive. FAT positive samples were sent to NRL to discriminate between wild and vaccinated strains using molecular biology techniques as previously described (Turcitu, 2010).
Statistical analyses were performed using GraphPad Prism version 6.01 software.

RESULTS AND DISCUSSIONS

Out of 6965 foxes planed to be hunted, 5448 (78.2%) were collected by hunters (Figure 1).

Out of these, 8% (n=436) were juvenile (Figure 2). Seventy three (1.33%) were positive by FAT. In a total of 5048 thoracic liquid examined, 1574 (31.18%) were positive by ELISA. Out of 1574 tested thoracic liquid, 142 (9%) were juvenile. Fifty five percents (n=5375) samples reacted positive to biomarker (tetracycline) detection (Figure 3).

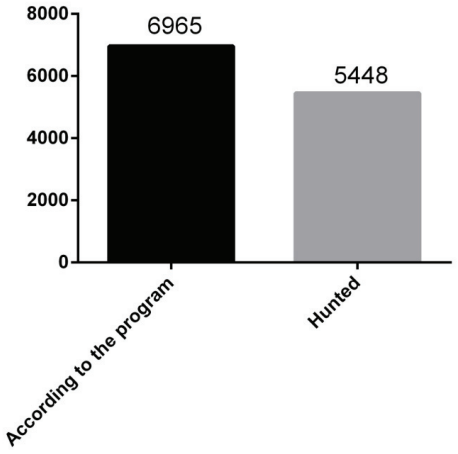

Figure 1. The number of the foxes planned to be hunted according to the program and the number of foxes achieved

Phylogenetic analysis demonstrated that all 73 field isolates from Romania found positive by RT-PCR belong to the classical rabies virus (genotype 1) and are all closely related. This shows that no vaccine-induced rabies cases occurred and all positive foxes were infected with wild rabies strains present in Romania (data not show).

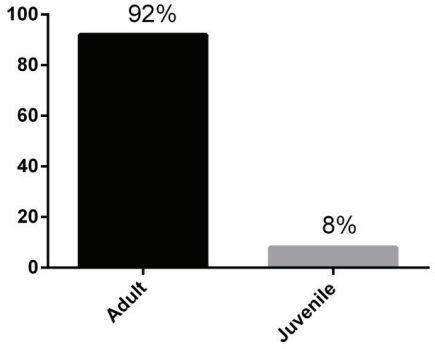

Figure 2. The age (%) of the hunted foxes

A foxes oral vaccination trial co-financed by the EU and the Romanian state budget has been conducted in 16 counties from the western part of Romania in spring and autumn 2011. From 2012 the OV programs were implemented throughout the Romanian territory according to the recommendations of the European Commission (European commission, 2002).

The efficacy of the OV campaigns in Romania was assessed by monitoring rabies prevalence in shot foxes, bait consumption and the immunisation rates. Oral vaccination campaigns were monitored from late July of the OV year to the following March by collecting head and serum samples from foxes. Autumn campaigns target adults and juveniles, while spring campaigns target mainly adults, because fox cubs are usually born from March to April, (Lloyd, 1980; Kauhala, 1996). We determined the age of all animals collected in 2014 in order to check the animal's effect on bait uptake and immunization levels as previously demonstrated (Bruyer, 2000).

Few studies have evaluated the effectiveness of OV according to the age of the target species (Bruyer, 2000; Rosatte, 2001). Tetracycline positivity rates were significantly higher than immunisation rates in both adults and young animals. There are several hypotheses may explain these discrepancies. First, the bait envelope may be ingested while the core containing the vaccine is not. Brochier et al. (1996) postulated that cubs may chew the baits without puncturing the vaccine capsule. When they are not hungry, foxes hide the baits in order to eat them later, which lead to inactivation of the live attenuated vaccine (Bachmann, 1990).

Fluorescence in teeth may be seen with an origin other than tetracycline from vaccine baits. Other possible sources of tetracycline include placental remnants from cows treated with tetracycline for infections associated with retained placentas, from chicken farm or from fish farms where tetracycline may be used (European commission, 2002). Foxes feed their cubs by regurgitation, and while regurgitated baits still contain tetracycline, the vaccine strain is destroyed by gastric acidity. Contact between the vaccine suspension and the oropharyngeal mucosa may sometimes be insufficient for immunization. The production of antibodies may be transient or absent, or

reach a low titer. In this last case, the sensitivity of the test used is a critical factor while the tetracycline gives life-long teeth deposits (European commission, 2002).

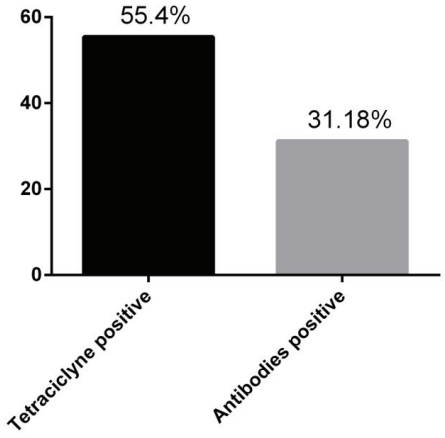

Figure 3. The percentages of the tetracycline positive samples and antibodies against rabies positive samples in hunted foxes

International organizations also recommend that ‘‘all rabies virus isolated should be typed in areas where attenuated rabies virus vaccines are used, in order to distinguish between vaccine and field virus strains’’ (European commission, 2002). Phylogenetic analysis demonstrated that all 73 field isolates from Romania found positive by hnRT-PCR belong to the classical rabies virus (genotype 1) and are all closely related. This shows that no vaccine-induced rabies cases occurred and all positive animals were infected with wild rabies strains present in Romania in 2014.

Vaccination will be succeeding in decreasing the outbreaks and eradicating the disease only if a sufficient percentage of population can be immunized. In a program aimed at free ranging wild animals, the proportion of the population which accept baits and the proportion of the individuals which will be protected from the rabies are essential features (Bachmann, 1990).

CONCLUSIONS

Romania has implemented a rabies control program since 2011.

Immunization rate and biomarker detection from the present ORV study suggest that distribution of vaccine in the field not have been too effective. Therefore additional research is needed to increase knowledge related the factors affecting the success or failure of wildlife oral rabies vaccination programs, such as effectiveness of the immune response in other species, like wild boars, jackals or even rabbits.

A continues monitoring and oral vaccination of foxes each year as well as the coordination and cooperation of vaccination programs between neighboring countries are very important.

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