# The field efficiency of oral rabies vaccination in the Lithuanian red fox population from 2006 to 2013

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**ABSTRACT**: The objective of the present study was to assess the efficacy of an oral rabies vaccination (ORV) program in the period from 2006 to 2013 based on epidemiological data (Incidence of confirmed rabies in red foxes), biological marker evaluation (presence of tetracycline [TTC] indicating bait uptake), and the assessment of seroconversion (based on ELISA for rabies antibodies) in red foxes in Lithuania. Results were compared between juvenile and adult red foxes, and between the spring (March to May) and autumn (October to December) campaigns. On average, 20 baits/km$^2$ were distributed over the entire territory of Lithuania. During the entire period of 2006–2013 1179 red foxes (10% of 11 829) were rabies-positive. While in 2006, 23.8% of tested foxes were rabies-positive, the incidence decreased to 2.4% in 2009, and 0.7% in 2011. Based on jaw bone investigation 78.1% of the foxes had ingested TTC from baits, 52.2% had seroconverted. The percentage of ELISA-positive red fox sera samples remained stable at 44.7–53.2% during both most recent ORV periods. The majority were adults (83%), 81.1 ± 8.54% of which were TCC-positive, and 73.8 ± 6.33% were TTC-positive juveniles. 52.5 ± 5.81% of adult red foxes had seroconversed, while 48.2 ± 4.51% of the young animals had a positive ELISA test in the years 2006 to 2013. This is in contrast to 2006 where only 29.5% of the young foxes were ELISA-positive. There were no significant differences between TTC- and ELISA-positive populations in different geographical regions of Lithuania.

**Keywords**: Baltic States; prevention; rabies; wildlife; TTC marker; ELISA seroconversion

Effective large-scale vaccination campaigns have successfully eliminated terrestrial wildlife rabies from most parts of Western Europe, where it primarily has been spread by the red fox (*Vulpes vulpes*) (Matouch et al. 2007). With strong efforts from Poland and other eastern European countries, the rabies-free zone has been expanded further to the east (Matouch et al. 2007; Cliquet et al. 2010). However, pockets of endemic wildlife rabies still remain in countries such as Russia, the Baltic States, and Belarus (Singer et al. 2009). The reasons for the persistence of rabies-positive foci include the high cost of vaccination campaigns, and the presence of other rabies vectors, which can make rabies control efforts more challenging in areas such as the Baltic States (Singer et al. 2009). While the red fox (*Vulpes vulpes*) remains the main rabies vector in Western Europe, the Raccoon dog (*Nyctereutes procyonoides*) and the arctic fox (*Alopex vulpes*) play an important role in the chain of infection in Eastern and Northern Europe (Wandeler 2008).

Oral rabies vaccination (ORV) programs targeting the red fox are currently carried out in whole territories or selected regions of 12 European Union (EU) Member States, and in neighbouring countries such as Russia, Ukraine, Belarus and in the Western Balkans (De Benedictis et al. 2012). Monitoring of vaccination is recommended in order to assess the effectiveness of ORV campaigns in the field. Post-vaccination surveys are based on direct and indirect parameters to evaluate vaccine efficacy that are preferably applied in combination, and include markers for bait uptake and population immunity rates, as well as the incidence of animals infected with rabies virus. Bait uptake in the target population is generally assessed by determining the presence of a biomarker that has been included in the vaccine formulation, such as

tetracycline (TTC). Monitoring of bait uptake is a useful tool that can be applied as an accompaniment to serological data (Lambot et al. 2001). For the assessment of the actual vaccine-induced immunity the presence of neutralising antibodies in serum is recognised as the most reliable parameter for assessing the efficacy of vaccination because it is closely correlated with protection against rabies infection. Thus, it is considered an efficient and direct estimate of vaccination efficacy in the target population (Wasniewski et al. 2013). Traditionally, recommended serological methods include the rapid fluorescent foci inhibition test (RFFIT) (Smith et al. 1996) and the fluorescent antibody virus neutralisation test (FAVN) (Cliquet et al. 1998). In addition, several enzyme-linked immunosorbent assays (ELISA) have also been developed to detect rabies-specific antibodies in animals, and most of them have been validated for their applicability with wildlife samples (Cliquet et al. 2003; Servat et al. 2008; De Benedictis et al. 2012). In 2010, eight out of the 11 EU countries implementing ORV programs adopted an ELISA method to monitor vaccine efficacy, with two using RFFIT and one using FAVN as gold-standards for comparison (De Benedictis et al. 2012).

In Lithuania ORV using attenuated live strains of rabies viruses was started in 2006, and an ELISA test has been used for detection of rabies-specific antibodies in wildlife target species including the red fox. The objective of the present study was to assess the efficacy of the ORV program during the 2006–2013 period by evaluating changes in the incidence of rabies-positive red foxes, and by tracking bait uptake marker detection and assessing immunological seroconversion in different subpopulations of red foxes in Lithuania.

## MATERIAL AND METHODS

**Oral rabies vaccination campaign**. During the years of 2006–2013 two annual rabies vaccination campaigns for red foxes were implemented in Lithuania during spring (March – May) and autumn (October – December). Vaccine baits were dispatched from light Cessna-type planes flying at an altitude of 200 metres either manually or using mechanical devices. The area of vaccination covered 65 000 km$^2$, which was divided into corridors of 500 metres. The average speed of the flight was 150 km/h and the average concentration of

baits km$^2$ was approximately 20 pieces. In total, 1 300 000 vaccine baits where distributed over the territory of Lithuania.

**Vaccine**. From 2006 until 2010 the rabies virus (RV) vaccine Lysvulpen (Bioveta a.s., Czech Republic) originating from a live SAD-Bern strain with a biological activity of $1.8 \times 10^6$ to $1.8 \times 10^8$ TCID50/bait was used. Starting in 2011 the RV vaccine Fuchsoral (IDT Biologika GmbH, Germany), a live SAD B19 strain (biological activity $10^6$ FFU/ml) has been used. One vaccination virus suspension dose (1.8 ml Lysvulpen SAD-Bern strain or 1.5 ml Fuchsoral SAD B19 strain) was pipetted each into an aluminium-plastic blister prior to sealing. Round, dark brown baits were prepared with a feed mixture attractive for foxes and other target animal species. Each bait contained 150 mg of tetracycline HCl. The baits with the vaccine containers inside were packed into cardboard boxes or polyethylene bags with an attachment grate. These were stored at –20 °C (monitored) in refrigerated lorries prior to use and over the course of the entire vaccination campaign.

**Biological data and sample collection**. The sex and age of all sampled foxes were recorded. The age of all animals was determined on the basis of a histological dental examination (young, less than 12 months; adult, more than 12 months of age) on microtome sections of the lower jaw bone.

For investigation of the epidemiological situation during the 2006–2013 period all rabies-suspected hunted (70–75%), road-killed (15–20%) and 5–10% of the foxes found dead were tested. The results were reported as rabies-positive or negative. Native brain samples were collected from foxes originating from different Lithuanian regions following the rabies control requirements approved by the State Food and Veterinary Service (SFVS). These requirements stipulate that all rabies-suspected pathological material (animal heads or brain samples) must be turned in for investigation at a district state veterinary service or to a regional veterinary laboratory. At such facilities positive rabies cases in native brain samples were diagnosed according to the methods standardised by the World Organisation for Animal Health (OIE) applying the fluorescent-antibody test (FAT) and the rabies tissue-culture infection test (RTCIT) (Dean et al. 1996; Bourhy et al. 1989).

Samples for the detection of TTC (lower jaw-bones) and rabies-specific antibodies were collected throughout the year, independent of vaccination

campaigns and under field conditions during the hunting period.

Blood samples of hunted foxes were collected directly from the heart or the aorta if possible, but mostly (75%) directly from the thoracic cavity. Blood samples were stored in a refrigerator at a temperature of approximately 4 °C for 24 hours. Serum was then separated by centrifugation and kept at –20 °C until used for antibody assays.

**Determination of TTC**. Lower jawbone (mandible) samples were removed and cleaned from surrounding soft tissues. They were stored in plastic bags at –20 °C. For analysis the mandible including the first premolar teeth was placed in an osteotome holder and 150–500 μm thick sections were prepared using a diamond microtome. Sections were rinsed in 70% ethyl alcohol, placed in a test tube and stored at –20 °C. For examination under a fluorescent microscope the samples were placed on a slide with a drop of glycerol. TTC presence was detected as a green/yellow fluorescence under ultraviolet light (excitation filter 380–425 nm, barrier 460 nm) (Johnston et al. 1987).

**ELISA**. Commercial standardised ELISA kits (Platelia$^{TM}$ Rabies II, Bio-Rad, Marnes-La-Coquette, France) consisting of 96-well plates coated with RV glycoprotein were used as previously described (Servat et al. 2007; Knoop et al. 2010). Samples were tested strictly according to the manufacturer's instructions. Briefly, all test serum samples were diluted one to 100, and 100 μl of each diluted sample were pipetted into each well of the microtitre plate, including positive (PC) and negative control (NC) sera. Plates were incubated for 1 h at 37 °C. Rabies antibody/glycoprotein G complexes were then detected by addition of 100 μl solution of Protein A-Peroxidase and purified bovine protein conjugate. The plates were incubated for 1 h at 37 °C, washed, and 100 μl of Tetramethylbenzidine chromogen solution 0.25% were added to each well. The plates were then incubated in the dark for 30 min at room temperature. The colourimetric reaction was stopped with 1N $H_2SO_4$. The results were expressed in optical density units, as determined by the Elx808 automatic microplate analyser (Bio-Tek, USA) at 450 nm. Serum antibody titres were expressed as equivalent units per ml (EU/ml) according to a standard curve established using the quantification standards (S1–S6) obtained by serial dilution of the high level control. The following controls were used – NC R3 (non-reactive TRIS-EDTA), PC R4a (0.5 EU/ml canine serum with anti-rabies

IgG), PC R4b (4 EU/ml canine serum with anti-rabies IgG). Samples with a titre > 0.5 EU/ml were considered positive/seroconverted (Cliquet et al. 2003).

**Statistical analysis**. Statistical analysis of the rabies epidemiological status and of TTC markers and ELISA-positive samples was performed using the limits of the coefficient of statistical reliability (*P*) and standard deviation (SD). Comparisons were evaluated between young and adult red foxes, and cohorts vaccinated during spring or autumn vaccination campaigns. In addition, results from the first period of ORV between 2006 and 2009 were compared to the second period between 2010 and 2013. Statistical analysis of positive investigation results was based on the absolute number of investigations and the quantity of positive samples expressed as percentages. The statistical analysis of the data was carried out using Prism3 software (Graph Pad Software, Inc., San Diego, USA).

# RESULTS

## Rabies epidemiology in Lithuanian red foxes between 2006 and 2013

During the entire period of 2006–2013 1179 red foxes (10% of 11 829) tested positive for rabies. Interestingly, during the first year of ORV campaigns in Lithuania in 2006, 23.8% (678/3681 tested) of the foxes sampled were rabies-positive. In the following three years the number of positive rabies cases in the tested fox population decreased to 2.4%, and after five years of ORV, in 2011, an incidence of 0.7% (5/628 tested) was determined. Concurrently, the number of red foxes suspected for rabies infection during that period decreased from 3681 in 2006 to 100 samples in 2013 (Figure 1). Comparative analysis of the first period (2006 to 2009) with 1154 rabies-positive foxes (11.9% of 9720 tested) and the second period (2010 to 2013) with 25 positive foxes (0.02% of 2094 tested) gave a statistically significant result (*P* = 0.0045), indicating an overall improvement in the epidemiological situation in the country.

## Bait uptake/TCC

Overall, 85–90% of hunted and tested red foxes were available for the TTC investigation, but 55–65%
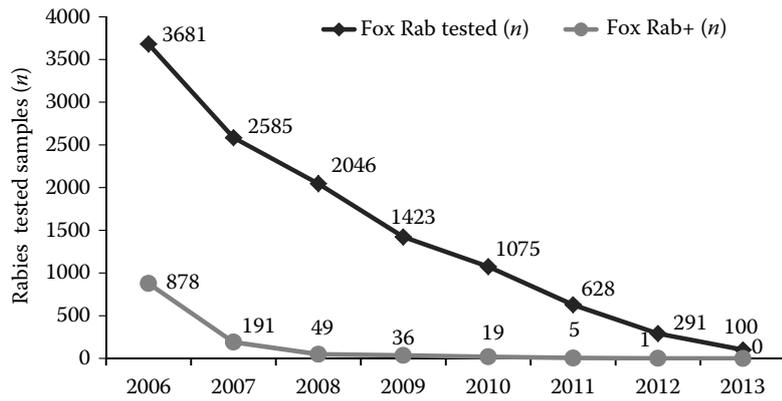
of the blood samples were of insufficient quality for the rabies antibody test. During the period from 2006–2013 jaw bones from 16 312 red foxes were investigated, and 78.1% (12 742/16 312) were considered TTC-positive. There was no significant difference between spring (78.5 ± 6.23%) and autumn ORV campaigns (79.5 ± 8.05%) for TTC-positive red foxes (SD 4.12%, Figure 2). Furthermore, the number of TTC-positive animals during the different ORV periods remained stable but increased during the spring periods from 63.4% (2006) to 94.5% (2012), and during the autumn periods from 69% (2008) to 92.6% (2013). A detailed geographical analysis of the bait uptake marker TTC revealed that the highest percentages of TCC-positive foxes originated from the Marijampole district (81.1 ± 5.25%), and the lowest from the Panevezys district (73.8 ± 4.88%) (Figure 4).

## Seroconversion

During the period from 2006–2013 serum samples from 4597 Red foxes where tested using ELISA, and 52.2% (2398/4597) were considered positive

with a seroconversion > 0.5 EU/ml. There was no significant difference between spring (51.1 ± 3.85%) and autumn (52.9 ± 6.33%) vaccination campaigns (Figure 2). However, there was a statistically significant ($P$ = 0.038) difference between the percentage of seroconverted animals in the spring of 2007 (37.2%) and the spring of 2010 (75%). Thereafter, the percentage of ELISA-positive red foxes sera samples remained stable at 44.7–53.2% during both recent ORV periods. The highest proportion of seroconverted animals was found in the Utena district with 53.7 ± 3.88%, and the lowest in the Vilnius district with 37.2 ± 3.20%.

## Efficacy of ORV in young and adult Lithuanian red foxes between 2006 and 2013

The majority of red foxes investigated were adults (83%), 81.1 ± 8.54% of which were TTC-positive, while 73.8 ± 6.33% were TTC-positive in the group of animals < 12 months old (Figure 3). In contrast, 52.5 ± 5.81% of adult red foxes had seroconverted, while 48.2 ± 4.51% of the young animals returned a positive ELISA test. Interestingly, in 2006 only
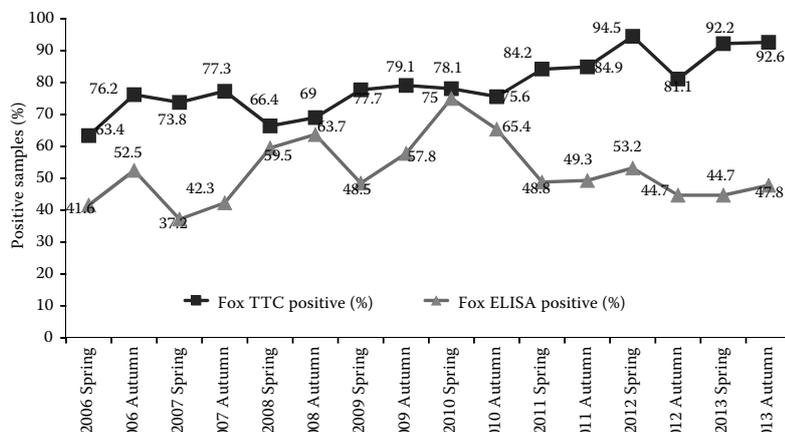


Figure 2. Frequency of tetracyclin marker (TTC) and seroconversion with antirabies antibodies (ELISA) in red foxes between 2006 and 2013 in Lithuania
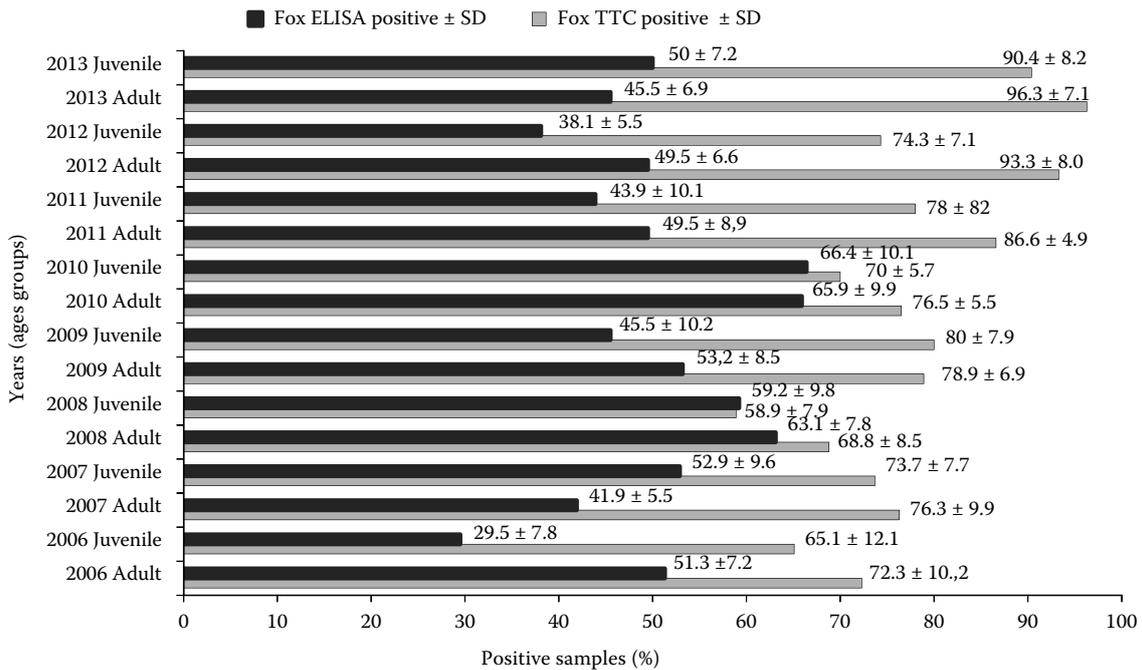
Figure 3. Frequency of tetracyclin marker (TTC) and seroconversion with antirabies antibodies in juvenile (< 12 months old) and adult (> 12 months old) red foxes between 2006 and 2013 in Lithuania

29.5% of the young foxes were ELISA-positive, and the proportion of positive serology increased by 25% in the following two years. Subsequently, however, in the years 2011–2013 the percentage of rabies Ab ELISA-positive sera samples was less than 50%, and with 5–11% ($P = 0.022$) differences between adults and young red foxes.

## DISCUSSION

Over the past three decades, 24 European countries have implemented ORV programs on their territories. Since 2000 ORV in several of these countries has been discontinued following successful rabies elimination, while new ORV programs have been initiated in the countries of Eastern Europe. To date, the total area covered at least once with vaccine baits between 1978 and 2010 encompasses approximately 1.92 million km$^2$. As a result of the ongoing implementation of ORV, the number of rabies cases reported annually in Europe steadily declined from 17 202 in 1978 to 7581 in 2010 (Freuling et al. 2013). In Lithuania, a 5-year ORV program was implemented from 1996 to 2000, covering between 4000 and 15 000 km$^2$, which corresponds to 6–23% of the territory of Lithuania. The three live rabies vaccine strains
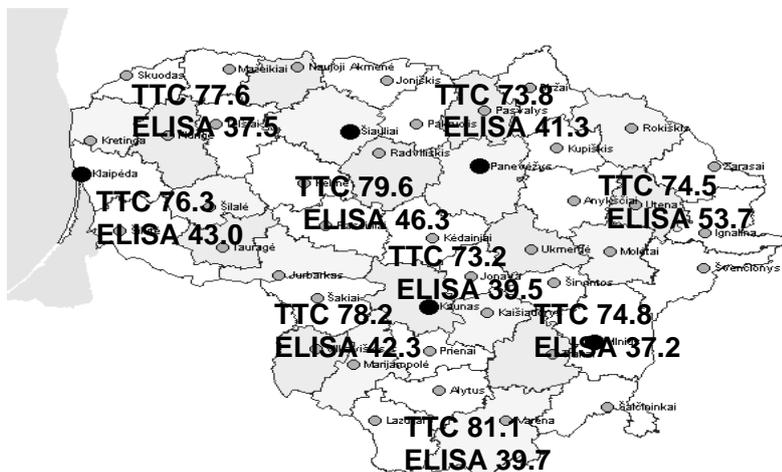


Figure 4. The geographical distribution of TTC/ELISA-positive samples (percentage) during the 2006–2013 ORV period on the territory of Lithuania

used (SAG1, SAD Bern and SAD B19) in a total of 900 000 baits were reported to be ineffective as an oral prevention of rabies in wildlife (Milius et al. 2004). Although ORV programs should ideally cover the entire affected area (Selhorst et al. 2005), initial programs in the Baltic countries failed to vaccinate the entire affected area (Cliquet et al. 2012). Specifically, initial ORV attempts in Latvia and Lithuania failed to efficiently control rabies, and were interrupted owing to budget constraints. Hence, subsequent ORV programs did not benefit from previous efforts and resources were wasted (Freuling et al. 2013). In contrast, ORV campaigns implemented between 2006 and 2013 covered the entire territory of Lithuania, which appears to have been an effective approach, as in 2012 there were just two reported rabies cases in raccoon dogs and one case in a red fox, and no rabies case was reported in Lithuanian wildlife in 2013. Likewise, the number of rabies cases has rapidly decreased in all three Baltic States since 2006; for instance, rabies has been nearly eradicated in Estonia since summer 2009 (Cliquet et al. 2012).

Levels of the TTC marker during the different ORV seasons were comparable for spring and autumn campaigns, and showed an increasing tendency (7–28%) throughout the period studied, suggesting a cumulative exposure to distributed baits (Rosatte et al. 2008). Juvenile red foxes appeared to have lesser access to baits, which confirms earlier studies with similar results (Rosatte and Lawson 2001). Bruyere et al. (2000) specified the difficulties in reaching fox cubs born between mid-March and April during baiting campaigns in the spring, especially when conducted in April instead of late May (Kauhala 1996). Autumn campaigns target both adults and juveniles. The interpretation of the diagnostic value of the TTC marker in the subpopulation of young individuals can be complicated due to a potentially less efficient uptake of the vaccine, as the capsule needs to be perforated. In addition, the TTC marker but not the antibody can be transmitted in milk during nursing (Brochier et al. 1994).

Therefore, the assessment of ORV efficiency is more reliable when based on classic serological studies of seroconversion. In the samples measured between 2006 and 2013 the proportion of seropositive red foxes was significantly lower (*P* = 0.016) than TCC positivity, both in adults and juveniles, with the difference being more marked in juveniles. Several hypotheses may explain the lower

level of seroconversion in juvenile foxes. Firstly, in the spring and early summer periods the majority of puppies are too small to consume vaccine baits that allow an efficient oral vaccination via the oropharyngeal mucosa. Further, maternal antibodies may prevent an active and sustained immunisation with a live oral vaccine (Hostnik et al. 2003). Clearly, the sensitivity of the ELISA used is a critical factor (Bruyere et al. 2000). The Platelia Rabies II kit (used in this study) was validated recently and performed well, i.e. with respect to specificity, sensitivity, and reproducibility (Servat et al. 2007). However, ELISA sensitivity compared to the standard serological tests of 36–82% for fox samples and 44–89% for domestic carnivores, respectively, was calculated depending on the method used. For fox field samples from ORV areas ELISA sensitivity compared to the Rapid Fluorescent Focus Inhibition Test (RFFIT) was only 32.4%. Therefore, ELISA cannot replace standard serological assays in followup investigations of ORV campaigns (Knoop et al. 2010). At the same time an ELISA test was developed for testing even highly hemolysed samples, because the fluorescent antibody virus neutralisation test, like other cell culture-based techniques, is too cumbersome for large scale screening, and too sensitive due to the cytotoxicity associated with poor quality samples (Servat et al. 2007). Thus, the relatively low proportion of foxes with seroconversion (37–72% of ELISA-positive serum samples) in the tested Lithuanian red fox population over the course of the long term ORV program may be directly associated with the poor quality of the tested serum samples. Nevertheless, it was an adequate alternative method to assess the rate of seroconversion during the oral vaccination period under challenging field conditions.

Importantly the epidemiological data collected during the 2006 to 2013 ORV period in Lithuania correlate with a decreasing incidence of rabies, not only in the red fox abut also in other wildlife (data not shown), including racoon dogs, albeit to a lesser degree. Although these results are encouraging, close contacts between red foxes and other potential rabies target species in Northern and Eastern Europe maintain a common infectious cycle. Certainly, future strategies with adapted vaccination campaigns for target species other than the red fox might be even more efficient in completely eliminating rabies on Lithuanian territory and preventing reintroduction from neighbouring countries.

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