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MAINTENANCE AND CONTROL OF THE VACCINATION BELT ALONG NEIGHBOURING RABIES INFECTED AREA

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The program of oral vaccination of wildlife started in 1988 in Slovenia and is based on our own, as well as experiences of other countries. Red foxes are the main reservoir of rabies in Slovenia. When the oral vaccination program had started on the whole territory of Slovenia in the year 1995, 1089 (28.75%) of tested animals were detected positive among wild and domestic animals. Four years later only 6 (0.5%) positive cases were detected among 1195 tested animals. The number of positive cases has been increasing again in 2001 to 135 cases. Between 2002 and 2008 the vaccination was been done only in the protection zone, i.e. 30 to 50 km wide belt along southern border with Croatia because no new rabies cases were found in the north-west region of Slovenia. When rabies was reintroduced in Italy in 2008 the vaccination is carried out again on the whole territory of Slovenia. To improve the vaccination campaign the stability of two vaccines was measured over 8 weeks. In both vaccines the drop of the virus titre was highest when baits were placed on sunlight, but in the shadow the virus was detected until 53 days of observation. The aim of this study is to summarise the current rabies status and to look for the best solutions in the vaccination campaign to come.

Key words: control, oral vaccination, rabies

INTRODUCTION

Rabies is a zoonotic disease of mammals, caused by infection with a negative-stranded RNA virus belonging to the Lyssavirus genus of the Rhabdoviridae family that can be divided into seven genotypes (Bourhy *et al*., 1999), mainly transmitted via the saliva through a bite from an infected animal (Niezgoda *et al*., 2002). In central and southeast Europe red foxes (*Vulpes vulpes*) are the main source of infection (Lontai, 1997). Vulpine rabies was established in Slovenia in 1973 for the first time. Results of phylogenetic analysis of a limited number of rabies virus isolates from Slovenia showed that isolates belong to genotype 1, groups of CE (Central Europe) and WE (Western Europe) (Bourhy *et al*., 1999). Control and eradication of rabies is a common strategy of European Union member states since the beginning of the 1980's (Pastoret, 1998).

Vaccination of wild animals against rabies was developed in the United States (Blancou, 1985; Bear *et al*., 1975) and was used for the first time in the field of Europe in Switzerland (Steck *et al*., 1982). Switzerland proposed the vaccination strategy that has been followed by other European countries, consisting of the compartmentalisation of the infected areas using natural or artificial barriers. In Switzerland the last rabies case was recorded in 1996. In EU countries industrially-produced baits have been distributed since 1980's, using live attenuated rabies virus strains known as SAD Bern, SAD B19, SAD-P5/88, SAG1, SAG2. The plastic capsule containing the fluid vaccine is incorporated in manufactured baits containing fish meal (Ahl *et al*., 2002).

Outbreak of rabies among racoon dogs and foxes started in 1988 in Finland, close to the south-eastern border of the country. Field vaccination campaigns started immediately using two campaigns per year, but since 1991 a single campaign was done. Thereafter, the country remained free of rabies, although the disease is endemic in Russia and Estonia (Metlin *et al*., 2008).

The Belgian programme of vaccination covered the entire infected area from 1989 to 1991, with 5 campaigns in total, leading to an 80% decrease of rabies cases. More restricted campaigns were conducted between 1992 and 1994 only along the borders of the country. Rabies cases were recorded again from 1994 to 1996, coming from a border residual focus. In 1996, the vaccination strategy was slightly modified and adapted to control rabies re-infected areas with a high density fox population. Two aerial vaccinations were carried out during the cold season (November and March, when the fox population density is the lowest in the year). Control of aerial distribution was improved by use of GPS (Global Positioning System) and reducing the distance between flight lines and baiting density was increased from 15 to 17 baits per km^2, supplemented by an additional den vaccination. Following this modification of the strategy and a close cross border co-operation with their French counterparts, rabies was efficiently controlled. The last rabies case was diagnosed in July 1999 in a 28 month old cow (Cliquet and Aubert, 2004).

In France, the peak in detected rabid animals with more than 4 200 cases was in 1989. The establishing natural barrier from the English Channel and Swiss border succeeded in stopping the westward and southward spread of the disease. During the vaccination between 1989 and 1996 the infected area was shifted towards borders resulting in a 99.7% decrease of rabies incidence. In France the last rabies case of vulpine origin was recorded in a cat in 1998. Part of the success story for eradication of rabies in Europe was the close co-operation between Belgium, France, Luxembourg and Switzerland to prevent cross-border contamination and improve their vaccination techniques (Aubert *et al*., 1986).

Italy carried out vaccination campaigns in the infected areas only when cases were recorded, since 1984. Rabies was eradicated in 1995, but after 13 years, in 2008 rabies was reintroduced again (De Benedictis *et al.*, 2008). Italy has been classified as rabies-free country since 1997. In October 2008, two foxes have been diagnosed with rabies in the Province of Udine, north-east Italy. One case of human exposure caused by a bite from one of the foxes has occurred and was properly treated. Since then rabies has been continuing and new rabies cases were reported in the north-east of the country in three regions; Veneto, Friuli-Venezia Giulia and Provincia autonoma di Trento. More than 180 animal cases have been recorded since 2008, mostly foxes, although other animals have been affected; badgers, deer, cats and donkeys. Most of the cases have been identified in the Veneto region where numbers have increased significantly in 2010 (Mutinelli, 2010).

Due to oral vaccination some European countries are now rabies-free in terrestrial animals; Finland (1991), Netherlands (1991), Italy (from 1997 to 2008), Switzerland (1998), France (2000), Belgium (2001) and Luxembourg (2001) (Potzsch *et al*., 2002) and last Czech Republic (Matouch *et al*., 2007). In other eastern and southern European counties where vaccination has not been practiced the number of reporting rabies cases is still high (Potzsch *et al*., 2002). Although the incidence of rabies has been greatly reduced with vaccination, zone between rabies positive area and rabies negative area should remain until the disease is present in neighbouring countries. The unexpected outbreaks have been reported in many countries (Müller, 1997; Matouch and Vitasek, 2007; De Benedictis *et al*., 2009). These problems were often resolved by increasing the number of vaccine baits (>30 baits/km^2), changing the timing and number of vaccinations, or improving and optimizing flight-line distances (Thulke *et al*., 1999).

This paper describes the course of rabies eradication in Slovenia and the stability of used vaccines in field conditions during the summer period.

MATERIALS AND METHODS

Following of the rabies epidemiological situation

Rabies was common in Slovenia in the dog population before Second World War, but the disease was eradicated by vaccination of local dogs during the 1950's. In 1973, the first sylvatic rabies was found in north-eastern part of Slovenia. In cooperation with the WHO surveillance centre in Tübingen, Germany, a field campaign with oral vaccination of foxes had started in September 1988. In the first period (from 1988 to 1992) the bait distribution was mainly done by local volunteer hunters, but later aerial distribution was used. Second period of eradication started in 1995 and has been successfully continuing until 2010. Rabies cases were decreased first in the north-west part of the country and later on along the border with Croatia a protection 30-50 km zone was formed.

According to Slovenian legislation, the veterinary organisations and hunters are obligated to send the samples from animals which had neurological symptoms of disease for laboratory examination. Veterinary administration has regulated additional monitoring of rabies in foxes with testing of 5 foxes per 100 km^2. An indirect immunofluorescent test (IIF) has been used for rabies diagnosis. It was performed on impression smears of Ammon's horns and cerebellum samples. The diagnostic technique was performed as is prescribed in the Manual of standards for diagnostic tests and vaccines (Dean *et al.*, 1996). Immuno-fluorescent conjugate produced by the Bio-Rad Laboratories from

France is used. Samples with doubtful results in IIF test are retested using virus isolation on murine neuroblastoma cells (Webster and Casey, 1996) or RT-PCR test (Black *et al*., 2002). The efficiency control of oral vaccination among foxes has been measured by active and passive monitoring with bait uptake control using the tetracycline biomarker detection method.

Oral vaccination

The program of oral vaccination is financed, inspected and planned by the Veterinary administration Republic of Slovenia. In Slovenia, when vaccination was started in 1988, the distribution sites were determined based on locations of previous outbreaks. However, due to an insufficient budget, at the beginning not every infected area was baited each year. The program was often interrupted, but it was done regularly on a national level since 1995. The basis of rabies eradication program in Slovenia consists of compulsory vaccination of all dogs and regular oral vaccination of foxes. Oral vaccination of foxes started on the whole territory in 1995. For the first two years only Fuchsoral vaccine, containing SAD B19 attenuated rabies strain (Steck *et al.*, 1982; Schneider and Cox, 1983), was in use. In the period from 1998 to 2004 the vaccine Fuchsoral has been laid down in the eastern part of the country and the Lysvulpen® vaccine (Vrzal *et al*., 1996), containing strain SAD Bern produced by Bioveta (Ivanovice) Czech Republic, has been laid down in the western part of country. Since 2006 only Fuchsoral vaccine has been used. Baits have been dropped by aircraft twice per year as a rule in September - October and May - June. In 1999 the area of 18 800 km^2 was covered (whole territory is 20 273 km^2), while the high mountain areas and urban areas were not included. Later, in 2000 when neighbouring countries Italy and Austria eradicated rabies, only the 12 500 km^2 belt on the south of the country has been treated. The average bait distribution density ranged from 20 - 25 baits/km^2. The baits were dispersed from a height of 300 – 500 meters. Pilots used the GPS navigation system for orientation and a computer-monitored discharge. A special computer program named FICO3J® (constructed by computer engineer Aleksander Modic) monitored the route, time of flying and calculated the density of distributed baits. During the period between 1995 and 2009, over 8 million baits were distributed across the country. Since 2009 after rabies reintroduction in Italy vaccination has been carried out on the whole territory of Slovenia again.

Bait stability trial

The thermo-stability of both vaccines used for oral vaccination (named now vaccine A and vaccine B) was tested under field conditions. The vaccine baits were placed on three different locations: in the open field, where direct sun was possible (Location 1), in high grass (Location 2) and in shadow under trees and bushes (Location 3), where direct sunlight was disabled. The distance between three locations was not longer than 100 m. This experiment was done during the summer period (from July to August). Ten baits of vaccine A and ten baits of vaccine B were placed on location 1, 30 baits of vaccine A and 30 baits of vaccine B were placed on location 2, and 50 baits of vaccine A and 50 baits of vaccine B were placed on location 3. Baits were laid down directly on the ground in a way that distances between baits were 2 cm minimum. Baits were covered with boxes made of wire to protected them against predators. The minimum and maximum temperature in each place was recorded daily using a contact thermometer. Thermometer sensors were placed in one ampoule of a bait to measure the real temperature in the bait. Consistence of baits matrix was observed and described on a daily basis. Baits were collected daily in the morning and stored at minus 70°C until virus titres were determined using microtitration technique (Aubert, 1996). Briefly, the bait matrix was removed and the liquid vaccine was placed into tubes and centrifuged at 2.500 x g for 15 min at 4°C. Ten-fold dilutions from 10^{-1} to 10^{-7} were prepared in mock plates (180 μL cell culture medium and 20 μL of sample) and 50 μL of each dilution was transferred onto 96-well tissue culture microplates (Nunc). Each dilution was transferred into three wells and BHK_{21} cell cultures were added and plates were incubated for five days at 37°C to determine the end-point dilution of the virus. After incubation the cell culture medium was removed and cells were fixed with 85% acetone at -20°C. The detection of rabies virus in the cell culture was carried out by an IIF. Briefly, conjugate (BIO-RAD Laboratories, France) were diluted 1:20 in PBS-T (0.01 M phosphate buffered saline plus 0.05% (v/v) Tween 20) and added on air-dried cell monolayer in 96-well microplates. After one hour of incubation at 37°C, the cells were rinsed with PBS-T and the cell monolayer was examined by UV light microscopy (Zeiss, Axiovert 25, Germany). The virus titre was than calculated as $TCID_{50}$/mL.

RESULTS

Rabies in Slovenia

During the period from 1995 – 2009 the 28 095 samples from different animal species were tested and 1.758 of them were rabies positive on IIF test. The majority of positive cases (94.6%) were found in wildlife, while among domestic animals only 5.4% positive cases were recorded. Among wildlife animals the foxes were rabies positive in 1 579 cases, martens in 31, badgers in 22, roe in 23, wild board 2, red deer in 1 and polecat 2. The highest number of rabies among domestic animals was detected among cats (53 rabies positive cases) followed by dogs (34 rabies positive cases), cattle (7 positive), sheep (3 positive) and one horse. Comparing the years before (period 1990 – 1995) and after the beginning of vaccination of the whole country (period 1996 – 2000) the incidence of rabies cases dramatically decreased from 2 989 to 411 rabies positive cases. In 2004, 2005, 2006 and 2007 rabies was confirmed in 2, 3, 2 and 3 animals respectively. In 2008, 55 and in 2009, 34 rabies cases have been registered (Table 1). All positive samples were collected in the vaccinated area close to the Croatian border. Only one case was found out of the vaccination area in 2009 in Kanal (near the border with Italy).

Table 1. Number of rabies positive animals and percentage of foxes marked by tetracycline in the vaccinated areas

Year	No. of tested animals	Total No. of rabid animals	Percentage (%)	No. of rabid foxes
1995	3 787	1 089	28.76	996
1996	2 285	247	10.81	208
1997	781	29	3.71	18
1998	1 382	14	1.01	14
1999	1 195	6	0.50	5
2000	1 509	115	7.62	104
2001	2 153	135	6.27	117
2002	1 495	15	1.00	14
2003	993	8	0.81	8
2004	1 612	2	0.12	2
2005	1 603	3	0.19	3
2006	1 796	2	0.11	2
2007	2 075	3	0.14	3
2008	2 619	55	2.10	51
2009	2 810	35	1.25	34
Total	28 095	1 758		1 579

Stability of vaccines in location 1 (open field)

The average maximum air temperature in location 1 during six days of the experiment was 29.8^{o}C. The maximum daily air temperature in the shadow was 34.4^{o}C. The maximum recorded temperature measured in the bait in the open placed area (location 1) was 46.3^{o}C, and minimum temperature during the night was 10.5^{o}C. After 24 hours all baits matrix of vaccine B placed in location 1 showed total destruction and the vaccine container and was not covered with matrix. In the case of vaccine A the bait matrix was partially destructed and the vaccine container was visible too. The experiment was finished after six days, because total destruction of matrix baits was observed. Results of the titrations of both vaccines are shown in Figure 1. The initial titre of rabies virus in vaccine A was 7.47 $\log_{10}$ $TCID_{50}$/mL and titer of virus in vaccine B was 7.57 $\log_{10}$ $TCID_{50}$/mL. After 24 hours the titre of rabies virus in vaccine A declined to 2.62 log 10 TID50, on day 2 were 0.71 log 10 $TCID_{50}$/mL, on day 3 the virus was not detectible on BHK $_{21}$ cell culture. After 24 hours the virus titre in vaccine B declined to 1.67 $\log_{10}$ $TCID_{50}$ and after 48 hours was not detectible. The colour of the liquid in bait vaccine B was changed in all capsules on day 2 and bacterial contamination with *Xanthomonas maltophilia* and *Enterococus sp*. was proved later.

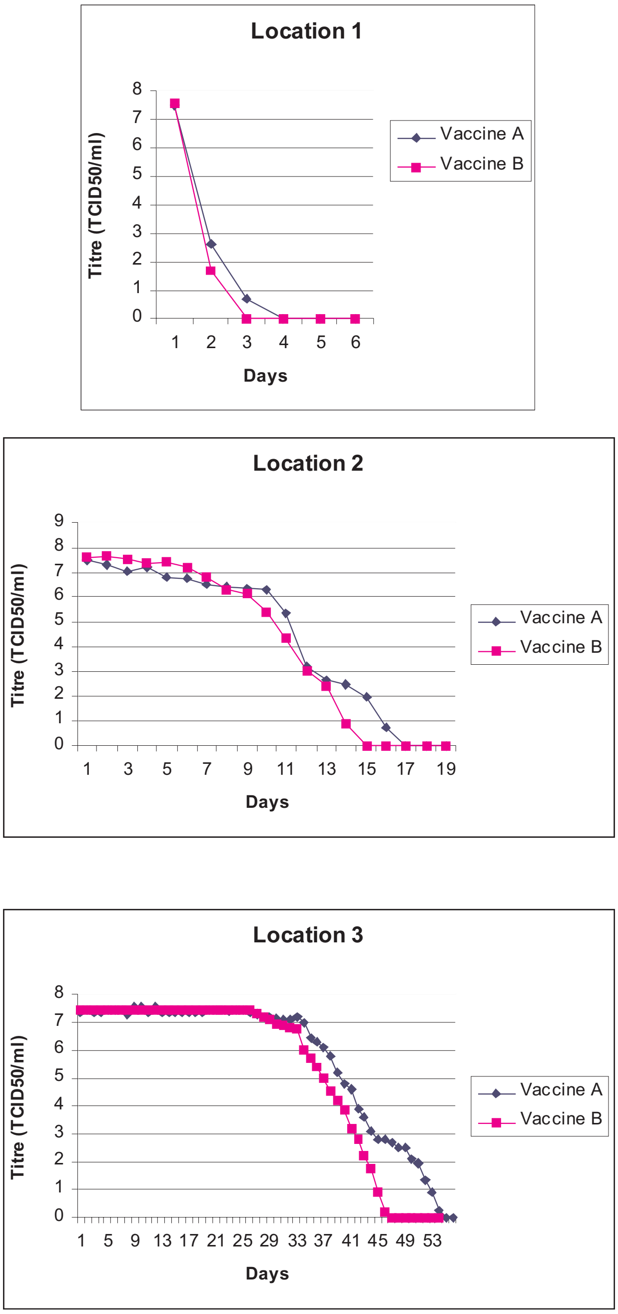

Figure 1. Vaccine titre stability in field conditions

Stability of vaccines in location 2 (high grass)

The titres of rabies virus in vaccine A and B placed in high grass (half shade conditions) has been measured during 18 days. The mean value of the maximum temperature was 36.6°C and minimum 14.2°C. In vaccine A the virus was detectable 16 days and 14 days in vaccine B. The virus titre in vaccine A declined by 50% during 10 days, but in vaccine B in 9 days. Bait matrix became damaged after 9 days in vaccine A, but in vaccine B the ampoules were exposed on the 3rd day of observation.

Vaccine stability in location 3 (in shadow under trees and bushes)

The titres of rabies virus in both vaccines were observed for 55 days. The mean value of the maximum temperature was 23.5°C and the mean value of minimum temperature during observation period was 13.8°C. Titre of rabies virus in vaccine A in location 3 was stable for 34 days and the titre declined by 50% on day 40. Virus in vaccine B was detectible up to 45 days, and the titre declined by 50% on day 39. Bait matrix in vaccine A remained intact during the observation period, but bait matrix of vaccine B became destructed on day 16 and ampoules with vaccines were visible.

DISCUSSION

Monitoring of the epidemiological situation in Slovenia is based on quite a number of collected samples each year. Hunters are motivated for sending samples to the laboratory with a reward for each hunted fox, paid by the government. Through all these years we are reaching the WHO recommendation with at least 8 animals per 100 km^2 to ensure successful control. The results of the vaccination between 1988 and 1992 have shown that the vaccination must be continuous, but the use of volunteers for vaccination purposes was unsatisfactory and the vaccination area did not cover enough territory. Due to the fox rabies vaccination the density of foxes population was enlarged, which should be considered when planning the vaccination. The increased number of detected rabies cases between 2000 and 2001 was linked to the insufficient number of baits per number of increased fox population, which has been notified also by other authors (Selhorst *et al*., 2006). In the period from 1992 to 1996 the territory of Slovenia was heavily affected by rabies, but a rapid decline of rabies was detected between 1995 and 1999, when the oral vaccination program using aircraft baits distributing system has been practiced on the whole territory (Table 1). In 1999 only 6 rabies cases were laboratory-confirmed, whereas only four years earlier 1 089 rabies cases were documented (181-fold decrease). The data in Table 1 shows that in Slovenia foxes are in most cases diagnosed as positive. Domestic animals infected with rabies represent only a small proportion (4.85%) of all rabid animals. This low percentage of rabies cases in domestic animals shows that the vaccination program of dogs and other domestic animals is useful. According to our experience, eradication of rabies among wild animals is a long lasting process. Efficient rabies eradication requires over-border cooperation which is still missing for the southern part of our territory. Despite all troubles, member states of EU are efficiently pushing rabies cases form the western part of EU towards the South-East (Selhorst *et al*., 2006). The minimum size of the vaccination area should be 5 000 km^2 (WHO and EU recommendations). If the precise location of the wildlife rabies cases is known, the size of the vaccination area is calculated by drawing a circle of 40 km radius around each case. The 27 EU member states have different rabies situation, 9 counties are free of rabies, Belgium (2001), France (2000), Luxenbourg (2001), Finland (1991), Netherlands (1991), Czech Republic (2004), Germany (2008) and Italy (1997 – 2008). All these countries have become rabies free as a result of wildlife oral vaccination programmes. All other EU infected countries are currently conducting oral vaccination programmes (Have *et al*., 2008).

Between 1988 and 1993 in Slovenia the baits were distributed partly using hunters' help plus by using helicopters and aircrafts. From this experience it turned out that it is impossible to control the actual bait distribution per km^2. Consequently the decision has been made in the second phase of vaccination in 1995 to start dropping the baits by using aeroplanes and GPS, which has proven to be of great value to the pilot as it enables a direct control of the dropped baits. The density of baits was increased from 18 bait/km^2 (1995) to 22 - 25 bait/km^2 (2010). When we used vaccines of two different manufacturers (Fuchsoral and Lysvulpen) the experiment of baits stability has been performed. It has been determined that the matrix of vaccine A was more stable comparing to the stability of matrix of vaccine B. From this experiment we can conclude that the vaccination cannot be carried out on days with daily temperatures over 30°C due to the rapid disintegration of the bait matrix. The vaccine sensitivity to high temperatures has been previously reported by Vos and Naubert (2002). The Lysvulpen vaccine stability in another study resulted that it is not very suitable for use in the summer time (Picard *et al*., 2006). Some authors (Masson *et al.*, 1999) reported that the recombinant vaccine VRG is remarkably thermo-stable, indifferent to high environement temperatures and is suitable vaccination in the summer months, which is necessary in the case of emergency vaccination. Daily temperatures during the spring time in Slovenia (end of May, start of June), when the vaccination campaign is performed, often reach 30°C. In this case the vaccine that is sensitive to high temperatures is not efficient enough. Balbo and Rossi (1988) have proven that foxes, vaccinated with baits, which have been exposed to high day temperatures, have a lower antibody titre. Baits of vaccine A in our study which were laid down in location 2 (high grass) was appropriate for fox vaccination for 9 days, and vaccine B only for 3 days due to the degradation of the matrix, although the virus titre both vaccines was above 6.0 $log_{10}/TCID_{50}/mL$ on day 9. Baits, distributed during the vaccination campaign in the forest shadow are considerably more efficient. The matrix of vaccine A in the experiment remained unchanged through the 55 days long testing period. On the other hand the capsules of vaccine B were less stable and became visible in the same period in 45% of tested baits. The percentage of forest areas exceeds 65% of Slovenian territory and the percentage of the agricultural area is approximately 28%, which means that there was not a considerable number of baits dropped in an open area such as in our test location 1. During the spring time many authors observed a lower uptake of baits comparing to the results obtained for the autumn season. In the spring time more than 70% of baits were taken within 3 to 6 days, but in autumn within 3 days. The reason for such a difference can be the abundace of food in the springtime (Pastoret *et al*., 1998).

The protected vaccination zone should be at least a 40 km belt with over 70% of vaccinated foxes, to reach satisfactory protection of rabies re-entry from infected area to rabies free area (Vos *et al*., 2001). When a considerably large number of rabies cases in Croatia was discovered there has been an increased number of rabies cases in Slovenia, as well (www.who-rabies-bulletin.org/Queries/Surveillance.aspx?Issue=2009_4). However, all the rabies positive animals were discovered in the vaccination zone not more than 15 km from the Croatian boarder. In year 2009 one rabid fox was found near the Italian border, 30 km out of the vaccination area, probably as a result of a not wide enough vaccination belt on the small border territory of Italy, Croatia and Slovenia. The narrowest vaccination belt in this region was between Trieste (Italy) and Jelovice (Croatia) with only 12 km distance. The high number of infected foxes in Croatia was detected over the years and represent a continuous risk for the reintroduction of rabies to our territory. The objective of the buffer zone in Slovenia is to maintain an adequate immunity in the fox population in order to prevent rabies epidemics in Slovenia and neighbouring countries as Italy and Austria. Occasionally, administrative borders may constitute barriers to the movement of foxes, but in most situations vaccination zones need to be defined and vaccination campaigns synchronised across administrative borders. A nice example of across borders cooperation was in case of reappearance of rabies in Italy on the border with Slovenia and Austria in 2008 (De Benedictis *et al.,* 2009). An emergency vaccination plan for Italy, Slovenia and Austria was made and the vaccination area in Slovenia was reconsidered on whole territory again. Italy implemented in 2008 local vaccination with a protected zone on infected areas (Italy has rabies-free status and the last campaign was carried out in 2004), two times per year in the three provinces of Udine, Goricia and Trieste close to the border of Slovenia. In order to avoid the spread of sylvatic rabies from Slovenia and Italy to Austria also in Austria an emergency vaccination campaign has been carried out. The recent example of rabies introduction into Italy clearly demonstrated the need for rabies free countries to maintain rabies expertise and an effective disease surveillance both for domestic and for wildlife.

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OČUVANJE I KONTROLA VAKCINALNOG POJASA OBLASTI U KOJIMA JE RASPROSTRANJENO BESNILO

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SADRŽAJ

Program peroralne vakcinacije divljih životinja je započet u Sloveniji 1988. godine i zasnovan je na domaćim iskustvima i iskustvima iz drugih zemalja. Cilj ovog istraživanja je bio da se utvrdi trenutna rasprostranjenost besnila i da se pronađu najbolja rešenja u predstojećem programu vakcinacije. Glavni rezervoar besnila u Sloveniji su crvene lisice. Kada je program ispitivanja efekata peroralne vakcinacije započet na teritoriji cele Slovenije, 1995. godine, 1089 (28,75%) testiranih domaćih i divljih životinja je bilo pozitivno na besnilo. Četiri godine kasnije, od 1195 testiranih životinja detektovano je samo 6 (0,5%) pozitivnih slučajeva. Broj pozitivnih slučajeva je opet porastao 2001. godine na 135. Između 2002. i 2008. godine vakcinacija je obavljena samo u zaštićenoj zoni, u 30 do 50 km širokom pojasu duž južne granice sa Hrvatskom, zato što nije bilo novih slučajeva besnila u severo-zapadnom regionu države. Kada se besnilo ponovo pojavilo u Italiji, 2008. godine, vakcinacija je opet obavljena na celoj teritoriji Slovenije. Radi poboljšanja efekata vakcinacije, merena je stabilnost dve vakcine tokom 8 nedelja. U obe korišćene vakcine pad broja virusnih čestica je bio najveći kad je mamac bio izložen sunčevoj svetlosti dok je u senci virus detektovan i do 53. dana posmatranja.