MAINTAIN AND CONTROL OF VACCINATION BELT ALONG NEIGHBOURING RABIES INFECTED AREA*

OHRŽAVANJE I KONTROLA VAKCINACIONOG POJASA DUŽ SUSEDNE POVRŠINE INFICIRANE BESNILOM

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The programme of oral vaccination of wildlife started in 1988 in Slovenia and is based on our and the experiences of other countries. Red foxes are the main reservoir of rabies in Slovenia. When the oral vaccination programme started in 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 been increased again in 2001 to 135 cases. Between 2002 and 2008 the vaccination was done only in the protection zone, a 30 to 50 km wide belt along the southern border with Croatia because no new rabies cases were found in the north-west region of the country. When rabies was reintroduced in Italy in 2008 the vaccination was carried out again in the whole territory of Slovenia. In order 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 the highest when baits were placed in the sunlight, but, in the shadow, the virus was detected until day 53 of observation. The aim of this study is to summarise the current status of rabies and to look for the best solutions in the next vaccination campaign.

Key words: rabies, oral vaccination, control
Introduction / Uvod

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 saliva through a bite from an infected animal (Niezgoda et al., 2002). In central and southeast Europe the red fox (Vulpes vulpes) is 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 a region of Slovenia showed that the isolates belong to genotype 1, groups of CE (Central Europe) and WE (Western Europe) (Bourhy et al., 1999). The control and eradication of rabies has been a common strategy of European Union member states since the beginning of the 1980s (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 in 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 1980s, using live attenuated rabies virus strains known as SAD Bern, SAD-B19, SAD-P5/88, SAG1, SAG2. A plastic capsule containing the fluid vaccine is incorporated into manufactured baits containing fish meal (Ahl et al., 2002).

An outbreak of rabies among racoon dogs and foxes started in 1988 in Finland, close to the south-east border of the country. Field vaccination campaigns were begun immediately with two campaigns per year, but since 1991 a single campaign has been 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. Then 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 the use of GPS (Global Positioning System) and reducing the distance between flight lines, and baiting density was increased from 15 to 17 baits per km², 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 effi-
ciently controlled. The last rabies case was diagnosed in July 1999 in a 28-month-old cow (Cliquet and Aubert, 2004).

In France, the peak for detected rabid animals with more than 4,200 cases was in 1989. The establishing of a natural barrier from the English Channel and the 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 of the eradication of rabies in Europe was the close cooperation between Belgium, France, Luxembourg and Switzerland on preventing cross-border contamination and improving 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 a rabies-free country since 1997. In October 2008, two foxes were 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 has been properly treated. Since then rabies has been continuing and new rabies cases have been 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 as well; badgers, deer, cats and donkeys. Most of the cases have been identified in the Veneto region where such 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 the Czech Republic as the last (Matouch et al., 2007). In other eastern and southern European counties where vaccination has not been practiced the number of reported rabies cases is still high (Potzsch et al., 2002). Although the incidence of rabies has been greatly reduced with vaccination, the zone between a rabies positive area and a rabies negative area should remain as long as the disease is present in neighbouring countries. Unexpected outbreaks have been reported in many countries (Müller, 1997; Matouch and Vitasen, 2007, De Benedictis et al., 2009). These problems were often resolved by increasing the number of vaccine baits (>30 baits/km²), changing the timing and the 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 the used vaccines in field conditions during the summer period.
Rabies was common in Slovenia among dog populations before the Second World War but the disease was eradicated by the vaccination of local dogs during the 1950s. In 1973, the first sylvatic rabies was found in a north-east part of Slovenia. In cooperation with the WHO surveillance centre in Tübingen, Germany, a field campaign with oral vaccination of foxes started in September 1988. In the first period (from 1988 to 1992) the bait distribution was mainly done by local volunteer hunters, but aerial distribution was later used. The second period of eradication started in 1995 and successfully continued until 2010. Rabies cases were decreased first in the north-west part of the country and later along the border with Croatia a 30-50 km protection zone was formed.

According to the Slovenian legislation, veterinary organisations and hunters are obligated to send samples from an animal which had neurological symptoms of the disease for laboratory examination. The Veterinary Administration has regulated additional monitoring of rabies in foxes with the testing of 5 foxes per 100 km². An indirect immunofluorescence test (IIF) has been used for rabies diagnosis. It was performed on impression smears of Ammon’s horn and cerebellum samples. The diagnostic technique was performed as it 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 was used. Samples with doubtful results in the IIF test are retested using virus isolation on murine neuroblastoma cells (Webster and Casey, 1996) or with the 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 / Oralna vakcinacija

The programme of oral vaccination is financed, inspected and planned by the Veterinary Administration of the 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 programme was often interrupted but it has been done regularly at nation level since 1995. The basis of the rabies eradication programme in Slovenia consists of compulsory vaccination of all dogs and regular oral vaccination of foxes. Oral vaccination of foxes started in the whole territory in 1995. During the first two years only the Fuchsoral vaccine which contains the 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 was placed in the east part of the country and the vaccine Lys-vulpen® (Vrzal et al., 1996) containing strain SAD Bern produced by Bioveta, Ivanovice, Czech Republic was placed in the west part of the country. Since 2006, only the 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 an area of 18,800 km² was covered (the entire territory is 20,273 km²), while the high mountain and the urban area was not included. Later in 2000 when neighbouring countries Italy and Austria eradicated rabies, only the area of a 12,500 km² belt in the south of the country was vaccinated. The average bait distribution density ranged from 20 - 25 baits/km². 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 the rabies reintroduction in Italy, vaccination has been carried out in the whole territory of Slovenia again.

Bait stability experiment / Eksperiment stabilnosti mamec

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 cut-grassing places, where direct sun was possible (Location 1), in the high grass (Location 2) and in the shadow under trees and bushes (Location 3), where direct sunlight was disabled. The distance between the 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 in location 1, 30 baits of vaccine A and 30 baits of vaccine B were placed in location 2, and 50 baits of vaccine A and 50 baits of vaccine B were placed in location 3. Baits were laid down directly on the ground in a way that distances between baits were at least 2 cm. Baits were covered with boxes made of wire to be protected against predators. The minimum and maximum temperature in each place was recorded daily using a contact thermometer. Sensors of the thermometer were placed in an ampoule of bait to measure real temperature in the bait. The 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 a microtitration technique (Aubert MFA., 1996). Briefly, the bait matrix was removed and the liquid of the 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 tree wells and BHK₂₁ cell cultures were added and plates were incubated for five
days at 37°C to determine the end-point dilution of the virus. After the incubation period, the cell culture medium was removed and cells were fixed with 85% acetone at -20°C. The detection of the rabies virus in 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 the 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 then calculated as TCID$_{50}$/ml.

Results / Rezultati

Rabies situation in Slovenia / Situacija sa besnilom u Sloveniji

During the period from 1995 – 2009, 28,095 samples from different animal species were tested and 1,758 of them were rabies positive in the 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, 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 horses (1 positive). Comparing the years before (period 1990 – 1995) and after the beginning of the vaccination in 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 were 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.

Stability of vaccines in location 1 (open cut-grassing place) / Stabilnost vakcina na lokaciji 1 (otvoreno mesto sa išećenom travom)

The average maximum air temperature in location 1 during six days of the experiment was 29.8°C. The maximum daily air temperature in shadow was 34.4°C. The maximum recorded temperature measured in bait in the open placed area (location 1) was 46.3°C, and the minimum temperature during the night was 10.5°C. After 24 hours all baits matrix of vaccine B placed in location 1 showed total destruction and the vaccine container 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 the total destruc-
tion of matrix baits was observed. The results of the titrations of both vaccines are shown in Figure 1. The initial titre of rabies virus in vaccine A was $7.47 \log_{10} \text{TCID}_{50}/\text{ml}$ and the titer of virus in vaccine B was $7.57 \log_{10} \text{TCID}_{50}/\text{ml}$. After 24 hours the titre of rabies virus in vaccine A decreased to $2.62 \log_{10} \text{TCID}_{50}$, on day 2 it was $0.71 \log_{10} \text{TCID}_{50}/\text{ml}$, on day 3 the virus was not detectible on BHK21 cell culture. After 24 hours the virus titre in vaccine B decreased to $1.67 \log_{10} \text{TCID}_{50}$ and after 48 hours it was not detectible. The colour of liquid in bait vaccine B was changed in all capsules on day 2 and bacterial contamination with *Xanthomonas maltophilia* and *Enterococcus sp.* was subsequently proved.

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

<table>
<thead>
<tr>
<th>Year / Godina</th>
<th>No. of tested animals / Broj ispitanih životinja</th>
<th>Total No. of rabid animals / Ukupan broj besnih životinja</th>
<th>Percentage / Procent (%)</th>
<th>No. of rabid foxes / Broj besnih lisica</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995</td>
<td>3,787</td>
<td>1,089</td>
<td>28.76</td>
<td>996</td>
</tr>
<tr>
<td>1996</td>
<td>2,285</td>
<td>247</td>
<td>10.81</td>
<td>208</td>
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<td>1997</td>
<td>781</td>
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<td>1.01</td>
<td>14</td>
</tr>
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<td>0.50</td>
<td>5</td>
</tr>
<tr>
<td>2000</td>
<td>1,509</td>
<td>115</td>
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</tr>
<tr>
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<td>2008</td>
<td>2,619</td>
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<td>51</td>
</tr>
<tr>
<td>2009</td>
<td>2,810</td>
<td>35</td>
<td>1.25</td>
<td>34</td>
</tr>
<tr>
<td>Total / Ukupno</td>
<td>28,095</td>
<td>1,758</td>
<td></td>
<td>1,579</td>
</tr>
</tbody>
</table>

Stability of vaccines in location 2 (high grass) / Stabilnost vaccine na lokaciji 2 (visoka trava)

The titres of rabies virus in vaccine A and B placed in high grass (half shade conditions) was measured during 18 days. The mean value of the maximum temperature was $36.6^\circ\text{C}$ and the minimum $14.2^\circ\text{C}$. In vaccine A the virus was detectable 16 days and 14 days in vaccine B. The virus titre in vaccine A decreased by 50 % during 10 days, but in vaccine B in 9 days. The bait matrix be-
came damaged after 9 days in vaccine A, but in vaccine B the ampoules were nak-
ed on the 3\textsuperscript{rd} day of observation.

Vaccine stability in location 3 (in shadow under trees and bushes) /
\textit{Stabilnost vakcine na lokaciji 3 (u senci ispod drveća i šbunja)}

The titres of rabies virus in both vaccines were observed for 55 days. The mean value of the maximum temperature was 23.5\degree C and the mean value of the minimum temperature during the observation period was 13.8\degree C. The titre of the rabies virus in vaccine A in location 3 was stable for 34 days and the titre decreased by 50\% on day 40. The virus in vaccine B was detectible up to 45 days, and the titre decreased by 50\% on day 39. The bait matrix in vaccine A remained intact during the observation period, but the bait matrix of vaccine B became destructed on day 16 and the ampoule with vaccines was visible.
The monitoring of the epidemiological situation in Slovenia is based on quite a big number of collected samples each year. Hunters are motivated to send samples to the laboratory with compensation for each hunted fox, paid by the government. Through all these years we are approaching the WHO recommendation with at least 8 animals per 100 km² to ensure successful control. The results of vaccination between 1988 and 1992 have shown that the vaccination must be continuous, but the use of volunteers for vaccination purposes was unsatisfactory in the long term and the vaccination area did not cover enough territory. Due to the fox rabies vaccination the density of the fox population was enlarged, which shall 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 populations, which has been noted 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 programme in the whole territory was practiced using an aircraft bait distributing system (Table 1). In 1999 only 6 rabies cases were laboratory-confirmed, whereas only four years previously a total of 1,089 rabies cases were documented (181-fold decrease). The data in Table 1 shows that in Slovenia foxes account for the 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 among domestic animals shows that the vaccination programme for dogs and other domestic animals is useful. According to our experiences during two decades the eradication of rabies among wild animals is a long lasting process. An efficient rabies eradication process requires trans-border cooperation which is still missing for the south part of our territory. Despite all the troubles, member states of the EU are efficiently pushing rabies cases from the west part of the EU towards the south-east (Selhorst et al., 2006). The minimum size of a vaccination area should be 5,000 km² (WHO and EU recommendations). If the precise location of a wildlife rabies case 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 situations, 9 counties are free of rabies, Belgium (2001), France (2000), Luxembourg (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 infected EU 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 using helicopters and aircraft. From this experience it turned out that it was impossible to control the actual bait distribution per km². Consequently the decision was 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 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 the vaccines of two different manufacturers (Fuchsoral and Lysvulpen) the experiment of bait stability was performed. It has been determined that the matrix of vaccine A was more stable comparing to the stability of the 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 showed that it is not very suitable for use in 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 temperatures in nature and appropriate for carrying out a vaccination in summer months, which is necessary in case of the need for emergency vaccination. Daily temperatures during spring time in our country (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 daily temperatures, contain lower antibody titre. Baits of vaccine A in our study which were laid down in location 2 (high grass) were appropriate for fox vaccination for 9 days, and vaccine B only 3 days because of the degradation of the matrix, although the virus titre was for both vaccines above 6,0 log$_{10}$/TCID50/ml on day 9. Baits, distributed during the vaccination campaign in forest shadow are considerably more efficient and the matrix of vaccine A in the experiment remained unchanged throughout the 55-day-long testing period. On the other hand, the capsules of vaccine B were less stable and became visible in the same period for 45% of the tested baits. The percentage of forest areas exceeds 65% of Slovenian territory and the percentage of agricultural area is around 28%, which means that there are not a considerable number of baits dropped in an open area such as in our test location 1. During spring time many authors observed a lower uptake of baits comparing to the results obtained for autumn. In spring time, more than 70% of the baits were taken within 3 to 6 days, but in autumn within 3 days, and the reason for such a difference can be the abundance of food in springtime for foxes (Pastoret et al., 1998).

The protected vaccination zone should be at least a 40 km belt with over 70% of vaccinated foxes to achieve satisfactory protection of rabies re-entry from an infected area to a rabies free area (Vos et al., 2001). When a considerably large number of rabies cases in Croatia was discovered there was 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 border. But in the year 2009 one rabid fox was found near the Italian border, 30 km out of the vaccination area, probably as a result of insufficiently wide vaccina
tion belts in 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 in distance. A high number of infected foxes in Croatia was detected over the years and this represent a continuous risk for the reintroduction of rabies into our territory. The objective of a buffer zone in Slovenia is to maintain an adequate immunity in the fox population in order to prevent rabies epidemics in Slovenia and also neighbouring countries such 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 good example of cross border cooperation was in the case of the 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 spread to the whole territory again. Italy implemented local vaccination in 2008 with a protected zone in 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, Austria has also been carrying out an emergency vaccination campaign. The recent example of rabies introduction into Italy clearly demonstrated the need for rabies free countries to maintain rabies expertise and effective disease surveillance both for domestic animals and for wildlife.

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Literatura / References


Ključne reči: besnilo, oralna vakcinacija, kontrola