

## Efficacy of Oral Vaccination in the Final Stage of Fox Rabies Elimination in Switzerland

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With 2 figures and 1 table

Received for publication August 12, 2004

### Summary

Subsequent to rabies vaccination campaigns, two well-established methods for the determination of the proportion of vaccinated foxes – the detection of tetracycline (TC) in bones and the detection of virus neutralizing antibodies (VNA) in thoracic fluids – were used and compared. Special emphasis was given to the effect of a new method of bait distribution at the den, which is primarily targeted at young foxes. The overall proportion of vaccinated animals estimated by TC was 60% as compared to 50% by VNA. In young foxes overall, significantly lower proportions of vaccinated animals (58% by TC and 40% by VNA) than in adult foxes (75 and 59%) were estimated with both methods. Low proportions of vaccinated young animals were found after spring (39 and 18%), but also after autumn vaccination (56 and 35%). In contrast, after den vaccination the level of vaccination of young foxes reached that of adult foxes. The theoretical implication of the successful elimination of fox rabies in Switzerland in spite of a relatively low overall proportion of VNA-positive animals is discussed.

### Introduction

Rabies is a fatal zoonosis of mammals caused by a rhabdovirus, which is present in most parts of the world. The European fox rabies epizootic of the past century, which originated in eastern Europe, reached Switzerland in 1967 and subsequently spread to the majority of the Swiss territory. The maximum number of rabies cases per year was recorded in 1978 with 1738 rabies cases, of which 72.7% were foxes as reservoir species and vector of the epizootic (Zanoni et al., 2000).

For the sake of public and veterinary health, the first field trial worldwide to eliminate fox rabies by oral vaccination was carried out in October 1978 in the Rhône valley in Switzerland (Steck et al., 1978). After the success of the trial, oral vaccination was gradually extended to the entire territory affected by the epizootic. Oral vaccination of foxes was carried out by manual distribution of vaccine-loaded baits (12–25 baits/km<sup>2</sup>) in endemic zones of fox rabies in spring and autumn (Kappeler and Wandeler, 2000).

After a steady decrease of rabies cases until 1990 (25 cases), a resurgence of the epizootic was observed in 1991 in the remaining endemic areas in north-western Switzerland. A continuous increase of rabies cases was observed during the following years, which was attributed to both a significant growth of the fox population and subsequent changes in the population dynamics. Young foxes had become an increas-

ingly important risk group for the persistence of rabies not observed before (Breitenmoser et al., 1995; Kaphegyi and Breitenmoser, 1995). To cope with this new situation, an additional oral vaccination campaign at the den in early summer was introduced in 1995, consisting of the distribution of six to 10 baits per active fox den (Breitenmoser et al., 2000). Since early 1997, no more indigenous cases of rabies have been observed in Switzerland.

To allow rapid estimation of the efficacy of oral vaccination in the vulpine population, tetracycline (TC) is added to the bait matrix as a marker, which will be incorporated in the bones of bait-consuming animals. However, the presence of TC, which is well separated from the liquid vaccine, is not a direct measure of immunization. Therefore, we also determined antirabies antibodies and compared estimates for vaccination coverage by the two methods for the final stage of rabies elimination in Switzerland and analysed the effect of the den vaccination on the young fox population.

### Material and Methods

#### Sampling of foxes

A total of 975 foxes, 845 from the vaccination zones in north-western and north-eastern Switzerland (Müller et al., 2000) and 130 foxes as a control sample from outside the vaccination area, sent to the Swiss Rabies Centre by hunters and game wardens for rabies diagnosis during the 1995/1996 vaccination period (19 June 1995 to 31 December 1996) were used for these analyses.

#### Age determination

Differentiation between the two age categories, young (< 1 year) and adult foxes (≥ 1 year), was carried out by visual inspection of incisival wear and confirmed by radiography of a lower caninus (Kappeler, 1991). The median birth date of the foxes was arbitrarily fixed on 1 March (Wandeler et al., 1974).

#### Grouping of foxes and oral vaccination schemes

To compare the effects of the different vaccination schemes ('classical' with spring and autumn campaign or 'den' with spring, den and autumn campaign) on young foxes, compared with adult animals, we distinguished four groups (Fig. 1): (i) Young foxes after the spring vaccination campaigns carried out from early March to late April (SPR in Fig. 1) until the

Month	Birth											
	M	A	M	J	J	A	S	O	N	D	J	F
Classical vaccination zone	Group 1: young foxes (SPR)						Group 2: young foxes (AUT)					
Vaccinations	SPR						AUT					
Den vaccination zone	Group 1 (SPR)			Group 3: young foxes (DEN)								
Vaccinations	SPR			DEN			AUT					
Both zones	Group 4: adult foxes (ADU)											
Vaccinations	SPR			DEN			AUT					

Fig. 1. Grouping of foxes in different zonal-seasonal vaccination groups. [SPR, spring vaccination; AUT, autumn vaccination; DEN, den vaccination (in italics for the group of the adult foxes, ADU, because they are pooled from two different vaccination zones, with and without den vaccination); (SPR), young foxes after the spring vaccination campaigns until the following vaccination campaign; (AUT), young foxes after the autumn campaign in the classical scheme; (DEN), young foxes after the den vaccination campaign in the den vaccination scheme; (ADU), adult foxes sampled from both areas (with and without den vaccination) throughout the year forming the control group].

following vaccination campaign (either the autumn campaign in the classical scheme, or the den campaign in the den vaccination scheme, respectively); (ii) young foxes after the autumn campaign carried out from early September to late October in the classical scheme (AUT in Fig. 1); (iii) young foxes after the den vaccination campaign carried out from mid-May to late June in the den vaccination scheme (DEN in Fig. 1); and (iv) all adult foxes sampled from both areas throughout the year were pooled into a single group (ADU in Fig. 1). All these animals had experienced two to several vaccination campaigns before.

#### Tetracycline detection

TC was revealed in 100–150  $\mu$ m thin sections of femur by UV transmission light microscopy (Laborlux 12; Leitz GmbH, Wetzlar, Germany) as described (Capt, 1981). Positive samples showed a fluorescent yellow to yellow-green colouration in at least one osteone or in the basal lamellae of the endosteum or periosteum.

#### Preparation of samples for serology

The two types of samples taken for serology from fox cadavers were the blood coagulate from the heart and thoracic fluid, if any. They were both treated with 50% ammonium sulphate final saturation for protein precipitation. After measurement of the protein concentration with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA), samples were calibrated to contain 10 mg protein/ml, corresponding to an approximate dilution of 1:5 compared with serum. This step was important for the reduction of cell culture toxicity of the samples.

To determine the quality of the samples from fox cadavers, blood was drawn from 18 foxes by intracardiac puncture directly after death. These foxes were then routed by post and the routine samples were taken for direct comparison.

To detect transplacental transfer of maternal antibodies, transsudates/exsudates derived from foetal inner organ pools

were sampled in some instances. To confirm the lactogenic transfer of antirabies antibodies and TC to fox puppies, milk was sampled from several vixens. TC analysis by CHARM Test II (Helnick, 1990) was carried out in the Research Unit Milk (Swiss Federal Dairy Research Station, Liebefeld, Switzerland). For serology, milk samples were treated by 50% ammonium sulphate final saturation for protein precipitation.

#### RFFIT for the detection of rabies VNA

A microtitre adaptation of rapid fluorescent focus inhibition test (RFFIT) in 96-well microtitre tissue culture trays was performed essentially as described (Smith et al., 1973; Zalan et al., 1979). The samples were serially diluted from 1:5 to 1:625 in BHK-21 cell culture medium. As control standard, a pool of human sera calibrated with the second international standard preparation for rabies immunoglobulin (Lyng, 1994) was used. A dose of  $10^4$  fluorescent focus forming units (FFU)/ml of SAD<sub>Berne</sub> (Street Alabama Dufferin; Steck et al., 1978) virus suspension was added to each sample or control dilution. After an incubation of 90 min at 37°C – 5% CO<sub>2</sub>, 0.2 ml of each dilution was then added to each of two wells of a microtitre tissue culture tray. Finally, 0.2 ml of a BHK-21 cell suspension containing  $10^6$  cells/ml was added to each well. The cells were then incubated at 37°C – 5% CO<sub>2</sub> for 25 h, washed and fixed after rabies virus inactivation by exposure to UV light. The presence of rabies virus was revealed using a goat anti-rabies/FITC conjugate (Swiss Rabies Centre). Ten microscopical fields per each well were read and fields containing at least one fluorescent cell were counted using a UV light microscope. The virus neutralizing antibody (VNA) titres were calculated according to Spearman-Kärber (Spearman, 1908; Kärber, 1931) by extrapolating the dilution of the sample reducing the number of fluorescent microscopical fields to 50% and international units (IU) were determined via standard control (3.1 IU/ml). Only tests with a titre of the control standard between 1:20 and 1:80 for 1 IU, a viral titre between 3000 and 30 000 FFU/ml and a cell control without fluorescent cells with a confluence of >90% were accepted for evaluation.

#### Statistical evaluation

For comparison of proportions of vaccinated animals, chi-squared statistics were applied (Fleiss, 1981). *P*-values < 0.05 were considered to be significant.

## Results

#### Overall findings of TC and VNA detection

Three of 130 foxes from non-vaccination zones (2.3%) were positive for TC but negative for VNA. The distance of their origin to the closest vaccination zone was 17 km in two cases and 42 km in the third. The last oral vaccinations in these areas had been carried out more than 10 years ago. Eleven of the foxes from non-vaccination zones (8.4%) were positive for VNA but negative for TC. The average distance of their origin to the nearest vaccination zone was 5 km (0.3–13.5 km).

From a total of 845 foxes (422 young and 423 adult) from the vaccination zone during the sampling period 1995/1996, 66% were positive for TC, whereas only 50% had detectable

VNA. A 36% of the TC-positive foxes were negative for VNA whereas 23% of the TC-negative foxes were positive for VNA (Table 1).

Of all adult foxes analysed with both methods, 75% were positive for TC whereas only 59% were positive for VNA. Of all young foxes analysed with both tests, 58% were TC positive but only 40% were positive for VNA. The differences between the proportions of TC- and VNA-positive animals were significant for both age groups.

For the comparison of the effects of the different vaccination schemes on young foxes, compared with adult animals (Fig. 1, Material and Methods), the group of young foxes after the den vaccination campaign (DEN) was pooled with the young foxes sent in after the autumn campaign in the den vaccination area, as neither the fraction of the TC- nor the VNA-positive animals increased significantly after the autumn campaign. Also the adult foxes sampled from both areas throughout the year were pooled (ADU), as there was only an insignificant increase in either TC or VNA percentages from the spring to the autumn campaign in this age class.

A 39% of the SPR group of young foxes were positive for TC whereas only 18% were positive for VNA and 14% were positive for both TC and VNA (Table 1). It is questionable to which extent this group may have been actively vaccinated during the spring vaccination campaign, which coincides with their birth/suckling period. In support of that notion, both TC and VNA were detected in two of three milk samples from lactating vixens. Furthermore, one of three foetal organ transsudates/exsudates was positive for VNA, which was also in accordance with the thoracic fluid of the corresponding vixen.

In the AUT group of young foxes, 56% were positive for TC whereas only 35% were positive for VNA and 29% for both TC and VNA. Higher estimates for the proportion of vaccinated animals were found in the DEN group of young foxes: 64% by TC, 50% by VNA and 43% by both TC and VNA (Table 1).

The highest proportions of either TC- or VNA-positive or both TC- and VNA-positive animals were found in adult foxes. The corresponding proportions were 75, 59 and 50% (Fig. 2). All the differences between the proportions of either TC- or VNA-positive animals in the SPR and AUT group on the one hand and the DEN and ADU group on the other were significant, except for the proportions of TC-positive animals in the AUT and the DEN group, respectively. In contrast, the

differences found between the DEN and the ADU group were not significant.

**Discussion**

A high discordance of 32% was found between the two tests used for the estimation of the proportion of vaccinated animals in favour of TC detection. Relatively high proportions of foxes were either positive for TC only (36% of TC-positive animals) or for VNA only (23% of TC-negative animals). Overall, the proportion of vaccinated animals estimated by TC was significantly higher, with an average of 25% (relative proportion), than estimated by VNA. Comparable discordances were also observed and discussed by Kappeler (1991) and Vuillaume et al. (1998). As TC and vaccine are separated physically in the bait, it seems obvious that the two markers (TC and VNA) are not strictly associated. Therefore, a possible explanation for the positive TC results in the absence of detectable VNA is the ingestion of the bait matrix without rupture of the vaccine container. Indeed, people out on a walk regularly reported the observation of intact vaccine containers in the field. Furthermore, also the relatively fast inactivation of the heat labile modified live rabies virus vaccine (Steck et al., 1982; Aubert et al., 2002) in the field may contribute to the discordance between TC and VNA. Other sources of TC (e.g. bovine placentas) cannot be excluded, but seem to be less

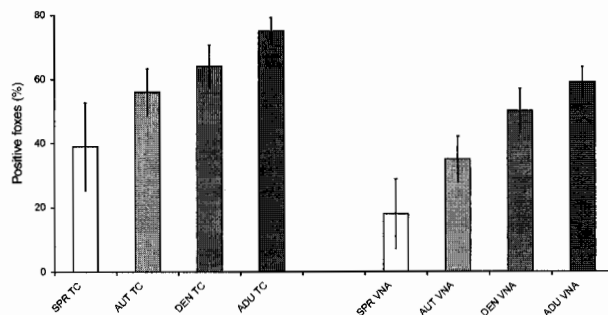


Fig. 2. Proportions of tetracycline and virus neutralizing antibody-positive foxes (SPR, AUT, DEN and ADU as in Fig. 1. TC, detection of tetracycline in bones; VNA, detection of virus neutralizing antibodies). The error bars represent the respective 95% confidence intervals.

Table 1. Detection of tetracycline and virus neutralizing antibodies in foxes

	TC pos <sup>a</sup> (%)	VNA pos (%)	VNA neg (%)	TC neg <sup>a</sup> (%)	VNA pos (%)	VNA neg (%)	VNA pos <sup>b</sup> (%)	TC pos (%)	TC neg (%)	VNA neg <sup>b</sup> (%)	TC pos (%)	TC neg (%)	Total
<b>Young foxes</b>													
SPR	19 (39)	7 (37)	12 (63)	30 (61)	2 (7)	28 (93)	9 (18)	7 (14)	2 (4)	40 (82)	12 (25)	28 (57)	49
AUT	98 (56)	51 (52)	47 (48)	77 (44)	11 (14)	66 (86)	62 (35)	51 (29)	11 (6)	113 (65)	47 (27)	66 (38)	175
DEN	127 (64)	86 (68)	41 (32)	71 (36)	14 (20)	57 (80)	100 (50)	86 (43)	14 (7)	98 (50)	41 (21)	57 (29)	198
TOT	244 (58)	144 (59)	100 (41)	178 (42)	27 (15)	151 (85)	171 (40)	144 (34)	27 (6)	251 (60)	100 (24)	151 (36)	422
<b>Adult foxes,</b>													
ADU	316 (75)	212 (67)	104 (33)	107 (25)	39 (36)	68 (64)	251 (59)	212 (50)	39 (9)	172 (41)	104 (25)	68 (16)	423
Total of all foxes	560 (66)	356 (64)	204 (36)	285 (34)	66 (23)	219 (77)	422 (50)	356 (42)	66 (8)	423 (50)	204 (24)	219 (26)	845

SPR, AUT, DEN and ADU as in Fig. 1. TC, detection of tetracycline in bones; VNA, detection of virus neutralizing antibodies.

<sup>a</sup>The proportions (percentages) of VNA-positive or -negative foxes (in italics) are relative to the corresponding TC results.

<sup>b</sup>The proportions (percentages) of TC-positive and -negative foxes (normal text) are relative to the total of all foxes of the corresponding group.

important in view of the small percentage of false-positive results found in non-vaccinated zones.

As an alternative explanation for this discordance, both technical (sensitivity of the serum neutralization assay used) and biological inefficacy of the exclusively serological detection of immunized animals must be considered. Protection in absence of VNA has been shown by several authors (Dietzschold et al., 1987; Sumner et al., 1991; Tollis et al., 1991; Herzog et al., 1992; Xiang et al., 1995). This possibility is interesting in view of models for rabies spread and control in fox populations (MacDonald and Voigt, 1985; Murray and Seward, 1992; Smith, 1995; Thulke et al., 1999; Smith and Cheeseman, 2002), according to which it seems unlikely to eliminate rabies in a high density population with a proportion of only 50% of immune animals. Therefore, the proportion of protected animals estimated by TC (66%) seems to be more realistic, although still at the lower bound for efficient control and the success of the campaigns observed in reality (Breitenmoser et al., 2000; Zanoni et al., 2000). Possibly, also this value is an underestimation of the proportion of vaccinated animals, when the proportion of animals, which were positive by VNA only (8% overall) is taken into consideration. Detection of VNA in absence of TC could either be explained by non-specific reactivity in the serum neutralization assay or by loss of the TC marker in the bone. The former seems rather unlikely in view of the starting dilution used (corresponding to an approximate dilution of 1:25 of sera), whereas the loss of TC-marked lamellae due to turnover of bone tissue, which is more pronounced in juvenile than in adult animals, seems plausible (Wandeler, 1991). In young foxes, also passive transfer of maternal antibodies could account for this finding.

We have shown that the proportion of vaccinated foxes is higher in adult than in young animals as estimated by either TC or VNA detection. This is compatible with the kinetics observed after repeated access to oral vaccination (Masson et al., 1996). The lowest proportion of vaccinated animals was found in fox cubs after spring vaccination. Still, in view of their reduced chance to consume baits during this vaccination period (Masson et al., 1999; Bruyere et al., 2000; Robertson et al., 2000), the proportion of both TC- and VNA-positive animals seems high (39% for TC and 18% for VNA). In a limited number of samples we have shown that antibodies can either be transferred transplacentally or by lactation from mother to cub (Xiang and Ertl, 1992; Blasco et al., 2001; Müller et al., 2001), although transplacental transfer was not confirmed in another study (Vos et al., 2003). Furthermore, also TC was detected in the milk of vixens. Therefore, it seems plausible that to some extent both TC and VNA could have been acquired passively by this age category. The limited half-life time of passive antibodies (Müller et al., 2002; Hostnik et al., 2003) combined with the low titres found in adult animals (data not shown) could explain the significant difference between the proportions of TC- and VNA-positive animals, respectively, found in this group (53% relative proportion). Alternatively, also the interference of maternal antibodies with active vaccination of fox cubs could attribute for this result (Vuillaume et al., 1998; Vos et al., 2001). Consequently, the autumnal oral vaccination campaign might be considered to be the first effective vaccination of young foxes, resulting in a proportion of vaccinated animals, which was not significantly higher than that after spring vaccination (corrected for multiple comparisons, not shown). As the autumn vaccination campaign takes place only after the

dispersal of young foxes from their natal home ranges has started, an early and effective vaccination of young foxes could be crucial to control rabies in a high population density situation. This underlines the potential importance of supplementary measures aiming at young foxes, such as den vaccination, additional or delayed conventional oral vaccination campaigns (Kaphegyi and Breitenmoser, 1995; Vuillaume et al., 1997, 1998; Masson et al., 1999; Selhorst and Müller, 1999; Bruyere et al., 2000; Have et al., 2004; but see also Vos, 2003). Our data confirm that den vaccination, although labour-intensive, is an important complement to classical oral vaccination campaigns, allowing to bring the critical age segment of young foxes to a level of protection comparable with that of adult foxes before dispersal starts in late summer.

### Acknowledgements

This work was supported by the Swiss Federal Veterinary Office (grant Nr. 012.96.2). The technical support of D. Brügger is greatly appreciated. We thank Prof. Dr M. Schällibaum (Research Unit Milk, Swiss Federal Dairy Research Station, Liebefeld, Switzerland) for performing the CHARM Test II. We also thank anonymous reviewers for their helpful comments, which have led to many improvements in the manuscript.

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