

## Comparative evaluation of specific ELISA and RFFIT antibody assays in the assessment of dog immunity against rabies

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### SUMMARY

Two techniques are currently used to evaluate the humoral immune responses to rabies vaccination: ELISA, which detects binding antibodies to viral antigens and the WHO reference rapid fluorescent focus inhibition test (RFFIT), which assays *in vitro* virus-neutralizing antibodies. In this study, we have comparatively evaluated antibody responses of dogs reared either in an experimental kennel or living in field conditions after vaccination with a cell culture-derived rabies vaccine. In experimental conditions, both ELISA and RFFIT techniques were well correlated. However, in field conditions, they yielded discrepant results particularly in evaluating the residual rabies immunity before vaccine administration and in identifying seroconverted dogs. After rabies vaccination in field conditions, while similar antibody titres and seroconversion rates were obtained using either technique, the discrimination of a given dog according to the seroconversion threshold depended on the assay. We concluded, that whereas in experimental conditions, ELISA and RFFIT were well correlated, in field conditions ELISA yielded upper estimates. Consequently, RFFIT, although a cumbersome test, should continue to be considered as the reference rabies antibody assay technique. A seroconversion threshold of 0.5 IU/ml should be cautiously considered and a higher threshold (1 IU/ml) could be more appropriate in the evaluation of rabies immunity in the field in order to marginalize the interfering factors.

### INTRODUCTION

Rabies is an encephalomyelitis which is almost always fatal with an underestimation of 60 000 human deaths each year [1]. Humans are mostly contaminated after being bitten by rabid animals among which, the dog is the most important vector and reservoir of the disease. Rabies remains a very serious public health concern, in most developing countries, especially in the Indian subcontinent, Africa and Latin America. This situation has led the WHO to initiate a number of programmes aimed at the control of canine rabies

by mass vaccination. These programmes have resulted in the control of enzootic dog rabies in many urban areas but have never been effective in eliminating the disease in the majority of these countries [2, 3].

Accumulating data from laboratory experiments and field observations clearly demonstrate that in animals, neutralizing antibodies are the most important immune effectors required for protection to a rabies challenge [4, 5]. For that reason, the WHO arbitrarily consider that an antibody titre of 0.5 IU/ml of sera is the threshold of seroconversion to a rabies vaccination. Furthermore, assays of virus-neutralizing antibodies are viewed as the easiest mean of evaluating the correct vaccine administration.

Several studies based on the evaluation of rabies antibody responses after mass vaccination of dogs

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yielded contradictory results. In Peru, 12 months after mass vaccination of dogs under field conditions with inactivated cell culture-derived rabies vaccine, 97% of dogs had rabies-neutralizing antibody titres of  $\geq 0.5$  IU/ml [6]. In contrast, a study conducted in Thailand concluded that the administration of a single dose of tissue culture-derived rabies vaccine was not able to maintain significant rabies antibody titres over 1 year. Moreover, levels of vaccine-induced rabies antibodies were already very low as early as 60 days post-vaccination [7]. In Tunisia, the immunogenicity in dogs of lamb brain-derived rabies vaccine produced locally, was evaluated in field conditions [8]. It was shown that 73% of dogs had seroconverted at 4 weeks post-vaccination at the peak of the immune responses, but this rate had fallen almost to the initial baseline level 7 months after vaccine administration.

These contrasting results in the assessment of rabies vaccination can be explained by a large variability in antibody responses from dog to dog and by several interfering factors, such as the vaccine brand, the route of vaccination, the age at vaccination, the health and breeding status of dogs. It is largely accepted that cell culture-derived vaccines are more efficacious than brain-derived vaccines. Moreover, the vaccine efficiency reported in industrialized countries in pet dogs, or in inbred dogs living in experimental conditions can hardly be extrapolated to that of dogs living in field conditions in developing countries. Such dogs are mostly poly-parasitized and suffer from malnutrition, two factors that may greatly affect their immune potency. Furthermore, the assessment of any vaccination strategy based on the assay of humoral immune responses may suffer from several technical variables. Two major techniques can be used for antibody assays, the rapid fluorescent focus inhibition test (RFFIT [9]) and ELISA-based tests [10–12]. For instance, one may question to what extent do binding antibodies tested by ELISA correlate with neutralizing antibodies as assayed by RFFIT. The latter technique is considered as the gold standard by the WHO, despite being more sophisticated and time consuming and suffering from some inter-laboratory variations. Nevertheless, the ELISA-based technique was recently adopted by the OIE (Office International des Epizooties) for the screening of sera for international pet movement schemes [13].

In the present study, sera from dogs living under experimental or field conditions were comparatively

evaluated for rabies antibodies by two different techniques: ELISA and RFFIT. The seroconversion threshold was also analysed, in order to evaluate its significance according to the assay technique, status of the animal, experimenter interpretations and vaccination status.

## MATERIAL AND METHODS

### Cells, viruses and vaccines

BHK-21 cell line was maintained in Dulbecco's Modified Eagle's Medium. The fixed PV (Pasteur virus) rabies strain was used to inoculate cell cultures in RFFIT antibody assays [14].

In experimental conditions, 2-month-old puppies from the local breed were recruited from the field, sheltered in an experimental kennel facility, vaccinated with Tetradog<sup>®</sup> (Merial, Lyon, France) to protect them against the major canine diseases (Parvoviruses, Distemper virus, Rubarth hepatitis, Leptospiroses), deparasitized and properly fed. In field conditions, no recommendations for the owners or intervention on dogs were made, except for blood sampling and vaccine administration.

One dose of the cell culture-derived rabies vaccine Rabisin<sup>®</sup> (Merial) was administered subcutaneously. We used a single-dose vaccination instead of the two doses recommended by the manufacturer in order to reproduce the actual conditions of mass vaccination of dogs in developing countries.

### Blood samples

A total of 306 sera were sampled from dogs and assayed for rabies antibodies by both ELISA and RFFIT techniques. Twenty sera were collected from four dogs maintained under experimental conditions and vaccinated with one dose of Rabisin. Dogs were bled at day 0 and then at days 35, 90, 180 and 365 post-vaccination. Under field conditions, 286 serum samples were collected from dogs vaccinated with Rabisin. These dogs were living in a rural area where no mass vaccination campaign against rabies had been performed during the last 3 years and for which owners stated that their dogs had not been vaccinated against rabies during that period. Dogs were bled before vaccination (0 day; 118 samples) and at days 60, 180 and 365 post-vaccination (with 54, 54 and 60 collected samples respectively). Sera were stored at  $-20^{\circ}\text{C}$  until required for use.

## Antibody assays

Antibody assays by ELISA used the Platelia Rabies kit (Bio-Rad, Marnes la Coquette, France), according to the manufacturer's recommendations. Results are expressed in EU/ml (equivalent units per ml of serum).

For rabies virus-neutralizing antibody assays we used the RFFIT technique as previously reported [9]. Briefly, various dilutions of serum samples were incubated for 24 h in the presence of the PV viral strain suspension infecting 70% of BHK-21 cells. Then, *in vitro* neutralization of the viral suspension was measured and titres were expressed in IU/ml (international units per ml of serum) using a WHO standard as the reference by determining the last dilution of serum sample which inhibited 50% of the initial fluorescent foci.

## Statistical analysis

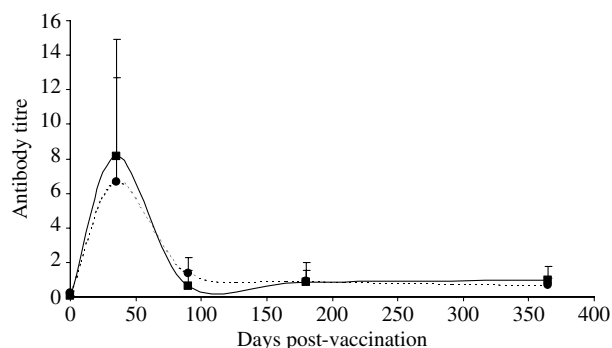
EpiCalc 2000, version 1.02 (Brixton, UK) was used for statistical analysis. The function calculates the difference between two means together with a 95% confidence interval, a *t* statistic and *P* value as described by Kirkwood [15], given the mean, standard deviation and sample size as determined by the Microsoft Excel. To calculate the *P* value for the *t* statistic test, the method uses the series summation as described by Abramowitz & Stegun [16]. For the determination of the correlation coefficients, measuring the degree to which two variables are linearly related, the Microsoft Excel was used.

## RESULTS

### Kinetics of rabies antibody titres measured by ELISA or RFFIT in dogs kept in experimental conditions

Four puppies from the local breed, aged 2 months were deparasitized, vaccinated with Tetradox and maintained for 8 months in an experimental kennel facility. These experimental dogs were considered adults at 10 months. At this age they had already lost any passively transferred rabies-specific maternal antibodies and they were vaccinated subcutaneously with one dose of Rabisin, a cell culture-derived rabies vaccine. Blood samples were taken at different time-points post-vaccination and tested for rabies antibody titres by ELISA and RFFIT.

At day 0, before vaccine administration, mean antibody titres were 0.22 EU/ml and 0.05 IU/ml by ELISA and RFFIT respectively. According to



**Fig. 1.** Kinetics of antibody responses of dogs vaccinated against rabies in experimental conditions. A group of four dogs were subcutaneously administered a single dose of cell culture-derived vaccine (Rabisin) at day 0. Dogs were bled at days 0, 35, 90, 180 and 365. The results represent the mean antibody titres expressed in IU/ml after RFFIT (—■—) assay and in EU/ml after ELISA (- -●- -).

RFFIT there was no residual maternal antibodies at the beginning of the vaccination protocol. However, in three out of four dogs, detectable antibody titres were recorded by ELISA, although below the extrapolated seroconversion threshold of 0.5 EU/ml. After vaccine administration, both RFFIT and ELISA yielded very close mean antibody titres (Fig. 1). At day 35 post-vaccination, antibody titres peaked at 8.1 IU/ml and 6.7 EU/ml with RFFIT and ELISA respectively, with all individual titres higher than 0.5 IU/ml and 0.5 EU/ml. At day 90, the mean antibody titres fell to 0.6 IU/ml and 1.4 EU/ml with RFFIT and ELISA respectively; at this stage, three out of four dogs had antibody titres <0.5 IU/ml by RFFIT and only one <0.5 EU/ml by ELISA. Similar results were obtained by day 180 post-vaccination, with identical mean antibody titres (0.9 IU/ml and 0.9 EU/ml); However, two out of four dogs had antibody titres <0.5 IU/ml and only one <0.5 EU/ml. By 1 year post-vaccination, means of 0.96 IU/ml and 0.7 EU/ml were found and one out of four dogs with a titre below the seroconversion threshold.

### Residual rabies antibodies before vaccination of dogs living in field conditions

To evaluate the efficacy of rabies vaccination in dogs living in field conditions, we targeted the whole accessible population in a representative rural area of the country. Based on questionnaires, it appeared that almost all dogs in this region had either never been vaccinated against rabies or not vaccinated during the previous 3 years. Therefore, we assessed the residual

Table 1. *Residual immune status of dogs living in field conditions. Before vaccine administration, all dogs were blood sampled and sera were assayed for rabies antibodies either by ELISA or RFFIT. A dog is considered seroconverted when its titre is  $\geq 0.5$  IU/ml by RFFIT and is presumed seroconverted when its antibody titre is  $\geq 0.5$  EU/ml by ELISA. The results are presented as totals or stratified for dogs younger or older than 2 years*

	Total		> 2 years		< 2 years	
	ELISA	RFFIT	ELISA	RFFIT	ELISA	RFFIT
Means	0.46	0.68	0.54	0.96	0.36	0.26
Medians	0.25	0.30	0.30	0.60	0.10	0.10
Seroconversion	29%	39%	31%	58%	27%	18%

humoral immune status of this dog population before vaccine administration. The mean residual antibody titres were 0.68 IU/ml by RFFIT and 0.46 EU/ml by ELISA (Table 1). The medians of antibody titres were, 0.3 IU/ml and 0.25 EU/ml by RFFIT and ELISA respectively. However, individual results significantly varied according to the age of dog: mean antibody titres as determined by RFFIT were 0.96 and 0.26 IU/ml for dogs older or younger than 2 years respectively and median titres of 0.6 and 0.1 IU/ml, in the same order. Using ELISA, 0.54 and 0.36 EU/ml mean titres and 0.3 and 0.1 EU/ml for medians, were found in dogs older and younger than 2 years respectively.

Before vaccination, 39% of dogs had an RFFIT titre  $\geq 0.5$  IU/ml. The total was 58% when the dogs were older than 2 years and only 18% when younger than that.

#### Kinetics of antibody titres tested by ELISA and RFFIT after rabies vaccination of dogs living in field conditions

Figure 2 shows that 2 months after vaccination with one dose of Rabisin, the mean antibody titres increased to 9 EU/ml by ELISA and to 4.3 IU/ml by RFFIT. At 6 months post-vaccination, the means fell to 2.1 EU/ml and 1.7 IU/ml by ELISA and RFFIT respectively. By 1 year post-vaccination, the means continued to decline reaching 1.1 EU/ml by ELISA and 0.82 IU/ml by RFFIT.

#### Kinetics of seroconversion rates tested by ELISA and RFFIT after rabies vaccination of dogs living in field conditions

The kinetics of seroconversion rates of dogs (with neutralizing antibody titres  $\geq 0.5$  IU/ml after RFFIT

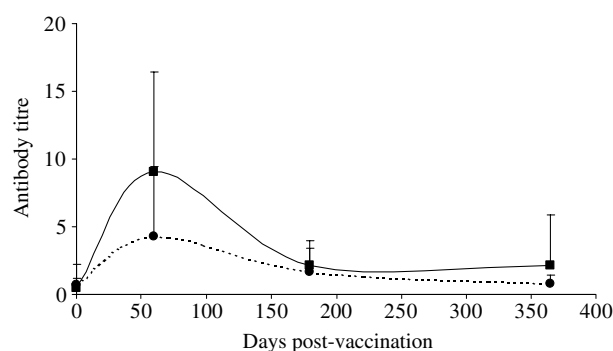
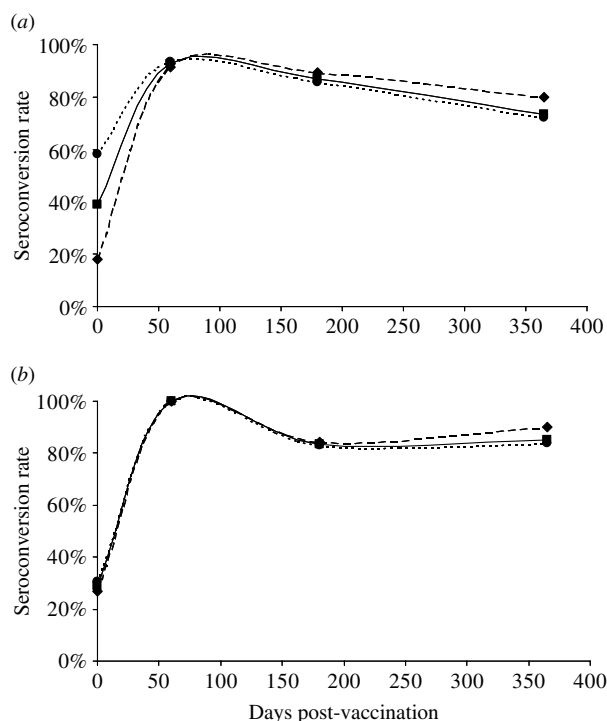


Fig. 2. Kinetics of antibody responses of dogs vaccinated against rabies in field conditions. A group of 198 dogs in field conditions were subcutaneously administered a single dose of cell culture-derived vaccine (Rabisin) at day 0. Dogs were bled at days 0, 60, 180 and 360. The results represent the mean antibody titres expressed in IU/ml after RFFIT (---●---) assay and EU/ml after ELISA (—■—).

assays) showed that, at 2 months post-vaccination almost all dogs (93%) have seroconverted, indicating that the vaccine brand was effective and vaccination was correctly applied (Fig. 3a). The seroconversion rates remained high at 6 months post-vaccination (87%) and fell slightly to 73% by 1 year. After vaccination, there was no significant difference of seroconversion rates according to the age of dogs.

If one considers a seroconversion threshold of 0.5 EU/ml as determined by ELISA, the extrapolated rates became: 100, 83 and 85% at days 60, 180 and 365 respectively (Fig. 3b). These figures are very close to what was obtained with RFFIT and they do not seem to be dependent on the age of the dogs at the time of vaccination.

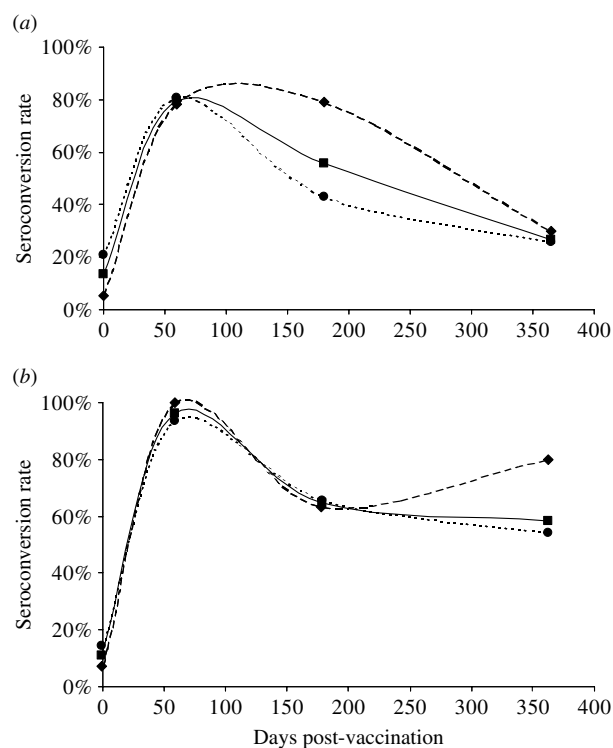
RFFIT may suffer from some subjectivity in results expression which may generate some ambiguous



**Fig. 3.** Kinetics of seroconversion rates of dogs vaccinated against rabies in field conditions. The same results as in Figure 2 are presented as seroconversion rates. A dog is considered seroconverted when its antibody titre is  $\geq 0.5$  IU/ml by RFFIT (a) and is presumed seroconverted when its antibody titre is  $\geq 0.5$  EU/ml by ELISA (b). The results are presented as totals (—■—) or stratified for dogs  $\leq 2$  years (—◆—) or  $\geq 2$  years (—●—).

conclusions with regards to seroconversion rates when using a threshold of 0.5 IU/ml. We, therefore, analysed the data using a threshold of seroconversion at 1 IU/ml (Fig. 4a). Before vaccine administration, 14% of dogs were above the threshold (21 and 5% for dogs older or younger than 2 years respectively). The rate reached 80% at day 60 after vaccine administration, but fell to 56 and 27% at 6 months and 1 year post-vaccination respectively. At 6 months post-vaccination the seroconversion rate was higher in younger dogs than in dogs aged  $>2$  years ( $P$  value 0.024); however, this difference at 1 year post-vaccination was no longer significant.

A similar analysis was carried out using ELISA and considering a seroconversion threshold of 1 EU/ml (Fig. 4b). When these results were compared to the rates given by RFFIT ( $\geq 1$  IU/ml) and excepting day 0, the ones given by ELISA were continuously slightly higher and without significant difference according to the age of dogs.



**Fig. 4.** Kinetics of rates of dogs vaccinated against rabies in field conditions with titres  $\geq 1$  IU/ml or  $\geq 1$  EU/ml. The same results of Figure 3 are presented as seroconversion rates but with a threshold of 1 IU/ml by RFFIT (a) and 1 EU/ml by ELISA (b). The results are presented as totals (—■—) or stratified for dogs  $\leq 2$  years (—◆—) or  $\geq 2$  years (—●—).

#### Comparative analysis of antibody responses measured by ELISA or RFFIT

Means of antibody titres measured by ELISA or RFFIT were compared using Student's  $t$  test, considering the null hypothesis that both techniques yield concordant results. We found that means were significantly different at day 60 ( $P$  value 0.0002) and at day 365 ( $P$  value 0.006).

The correlation coefficients (CC) of antibody titres obtained by either ELISA or RFFIT with each individual sample were also calculated. They showed large variations according to the time elapsed after vaccine administration (Table 2). Before vaccination, correlation was very weak (CC 0.29). After vaccination, it increased to 0.46 at 2 months and peaked at 0.69 at 6 months, then declined to 0.36 at 12 months.

According to the WHO recommendations it is acknowledged that reaching a rabies virus-neutralizing antibody threshold of 0.5 IU/ml is an indicator of seroconversion to a rabies vaccination. For that reason, we checked whether ELISA and RFFIT

Table 2. Correlation between antibody titres measured by ELISA or RFFIT in dogs living in field conditions at different time spans before or after vaccine administration

Day	ELISA	RFFIT	Student's <i>t</i>	D.F.	<i>P</i> value	Correlation coefficient
0	0.46	0.68	1.41	234	0.160	0.29
60	9.03	4.31	3.82	106	0.0002*	0.46
180	2.13	1.67	1.31	106	0.193	0.69
365	2.18	0.82	2.81	118	0.006*	0.36

Each sample was assayed simultaneously by RFFIT and ELISA and the means were compared by Student's *t* test (\* denotes the difference of value is statistically significant with the corresponding *P* value) and the overall correlation coefficient is determined.

techniques permit accurate discrimination of seroconverted dogs and ensure concordant results.

As a first step, we analysed the significance of the pre-vaccination residual humoral immune responses detected in dogs living in field conditions (at day 0). The seroconversion threshold levels were set at 0.5 EU/ml according to ELISA and 0.5 IU/ml with RFFIT (Fig. 5, Table 3). Hence, only 53% of dogs with titres  $\geq 0.5$  IU/ml by RFFIT also had ELISA titres  $\geq 0.5$  EU/ml. In addition, 33% of dogs considered as seroconverted by ELISA had RFFIT titres  $< 0.5$  IU/ml. Similarly, almost 60% of RFFIT seroconverted dogs had ELISA titres  $< 0.5$  EU/ml. Overall, only 30% of dogs with titres  $\geq 0.5$  IU/ml or  $\geq 0.5$  EU/ml, were concordantly classified as seroconverted by both tests. We, therefore, checked whether a higher threshold of ELISA (1 EU/ml) could better predict the seroconversion status of dogs with RFFIT titres  $\geq 0.5$  IU/ml. In such a situation and despite the small number of samples, the discrimination of RFFIT-seroconverted dogs was very poor (46%). Even if the ELISA threshold is increased to 2 EU/ml and compared to 0.5 IU/ml by RFFIT, the discrimination of seroconverted dogs remained very poor (50%). All these results show that in dogs without recent administration of rabies vaccination, binding antibodies detected by ELISA obviously do not correlate with rabies virus-neutralizing antibodies as determined by RFFIT.

Similar analysis was carried out at different time-points after vaccine administration. At day 60, when the seroconversion threshold was set at 0.5 EU/ml by

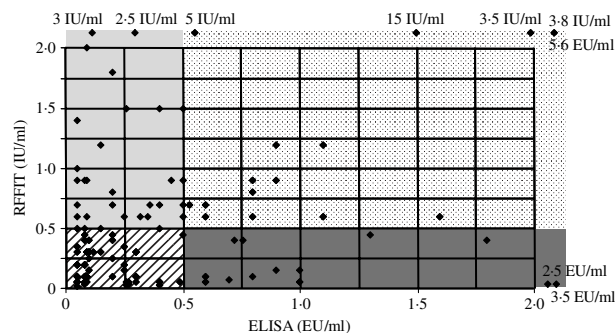


Fig. 5. Correlation between antibody titres measured by ELISA or RFFIT in blood samples taken from dogs living in field conditions before rabies vaccine administration. Samples inside the area (▨) are seroconverted by ELISA and by RFFIT. Samples inside the area (□) are not seroconverted neither by ELISA nor by RFFIT. Samples inside the area (▤) are seroconverted by RFFIT but not by ELISA. Samples inside the area (▥) are seroconverted by ELISA but not by RFFIT.

ELISA and compared to 0.5 IU/ml by RFFIT, the discrimination of seroconverted dogs was 93% and almost all dogs were accurately identified by both assays (Table 3). When ELISA thresholds were set at 1 or 2 EU/ml, the detection of RFFIT-seroconverted dogs increased insignificantly to 94% and 96% respectively. At day 180, when ELISA antibody titres were  $\geq 0.5$  EU/ml, invariably they were 89% and 78% in groups of dogs either seroconverted or not by RFFIT respectively. Invariably, the discrimination of RFFIT seroconversion by ELISA is  $\sim 95\%$  if the antibody titre is  $\geq 1$  or 2 EU/ml. By day 365 the situation had slightly improved, with ELISA technique at the threshold of 0.5 EU/ml, 80% of RFFIT seroconverted dogs were identified but still 40% of dogs with titres  $< 0.5$  IU/ml were assayed as seroconverted by ELISA. The discrimination rate of RFFIT-seroconverted dogs decreased to 83% and 89% when the ELISA titre of the dog was  $\geq 1$  or 2 EU/ml respectively.

## DISCUSSION

The most convenient method to assess the immune status induced by vaccination is to assay rabies antibodies. This assessment should be based not only on conclusions drawn from investigations carried out in experimental conditions or in healthy pet dogs, but it should also include dogs living in field conditions in developing countries. The latter dogs are frequently poly-parasitized and suffer from malnutrition, which

Table 3. Correlation of seroconversion rates of dogs living in field conditions as determined by either ELISA or RFFIT at different time spans before or after the vaccine administration

	Day 0		Day 60		Day 180		Day 360	
	RFFIT ≥0.5	RFFIT ≤0.5	RFFIT ≥0.5	RFFIT ≤0.5	RFFIT ≥0.5	RFFIT ≤0.5	RFFIT ≥0.5	RFFIT ≤0.5
ELISA ≥0.5	53 %	33 %	93 %	100 %	89 %	78 %	80 %	40 %
ELISA ≥1	46 %		94 %		95 %		83 %	
ELISA ≥2	50 %		96 %		95 %		89 %	

may greatly depress their immune responses and affect data interpretation. These dogs represent the vast majority of dogs targeted by the mass vaccination campaigns conducted by several rabies endemic countries.

When antibody titres induced by rabies vaccination of dogs kept in experimental conditions were compared to those obtained in field conditions, interesting findings emerged. At 6 months post-vaccination, surprisingly both virus-neutralizing antibodies (assayed by RFFIT) and binding antibodies (assayed by ELISA), appeared to be higher in dogs living in field conditions compared to those kept in experimental conditions. By 1 year post-vaccination, binding antibodies seemed to be still higher in dogs in field conditions, but neutralizing antibody titres were similar in both conditions. Since dogs vaccinated in experimental conditions were naive dogs, it is better to compare their immune response with dogs <2 years old living in field conditions, that have probably never previously been vaccinated against rabies. Hence, at 6 months post-vaccination, younger dogs living in field conditions gave binding and virus-neutralizing antibody titres which seemed to be slightly higher than those living in experimental conditions. By 1 year, only binding antibodies seemed to be higher in dogs living in the field. Several hypothesis could account for the observed difference of antibody responses between dogs living either in experimental or field conditions. (i) Field conditions may enhance the antibody responses of vaccinated dogs; such hypothesis seems to be unlikely, since it is largely accepted that in field conditions dogs frequently suffer from malnutrition and several parasitic and infectious diseases. However, one may not exclude the possibility that this enhancement can be associated with polyspecific antibodies which are able to bind to a large range of antigens [17]. (ii) Subclinical infection by rabies virus may occur in field conditions in countries endemic for rabies boosting the subsequent

post-vaccination immune responses [18–21]. Although, rabies endemicity can have various epidemiological consequences on viral transmission, subclinical infection is very poorly described in the literature and the extent to which it may enhance the residual immunity of dogs to rabies has yet to be thoroughly investigated. (iii) Cross-reacting antibodies induced by other infectious agents, associated with the poor health status of dogs in field conditions may interfere with the antibody assay technique. In addition, rabies vaccines produced on cell culture may contain cell contaminants, the latter can trigger the production of cell-specific antibodies by the vaccinated dogs [22]. ELISA plates are also coated with the rabies glycoprotein extracted from viral particles grown on cell culture. Cellular contaminants co-extracted with the glycoprotein may contribute to falsely amplify the antibody titre. This interference is probably insignificant when specific antibody titres of dogs are high (i.e. shortly after vaccination). However, it could significantly distort the results when antibody titres are rather low as in residual pre-vaccination immunity or at 12 months post-vaccination. This bias also seems to be dependent on the vaccination conditions: whereas in experimental conditions, ELISA and RFFIT are well correlated, in field conditions ELISA yields upper estimates. Consequently, RFFIT, although a cumbersome test, should continue to be considered as the reference rabies antibody assay technique.

Almost 60% of dogs >2 years old, living in field conditions were already seroconverted by RFFIT before the start of the vaccination protocol, despite the denial by the owners of any rabies vaccination during the previous 3 years. Similar results were clearly documented by a study carried out in Tanzania in which 49.4% of unvaccinated dogs were seropositive in rabies endemic areas and even 10.3% of unvaccinated dogs in a rabies-free island had an antibody titre ≥0.5 IU/ml using RFFIT [23]. In

another study in Ethiopia, 80% of unvaccinated dogs have detectable rabies antibodies by ELISA and RFFIT [24]. Several hypothesis may account for these results. (i) A consistent residual immunity to rabies vaccination, especially in older dogs, could be maintained over years in field conditions by continual exposure to rabies antigen or to cross-reacting antigens. (ii) The detected antibodies may have no role in protection to rabies infection: some unvaccinated dogs were seropositive by RFFIT, although they do not express any binding antibodies by ELISA. Therefore, although a seroconversion threshold of 0.5 IU/ml could be meaningful in dogs vaccinated under experimental conditions or in pet dogs kept in favourable conditions, it can be confusing in dogs vaccinated in field conditions, where multiple infections and numerous interfering factors bias the interpretation of antibody levels. Partly because of that, we think that a threshold of 1 IU/ml is preferable to predict the likelihood of protection against rabies infection and to marginalize the effects of interfering factors, in order to better weight the importance of the vaccination intervention in the induced immune responses.

The following analysis illustrates how discrepant the results might be depending on the antibody assay and the seroconversion threshold in dogs vaccinated in field conditions. When the antibody assay is ELISA and the threshold is set at 0.5 EU/ml, rabies vaccination appears rather very effective at 6 and 12 months post-vaccination, since the seroconversion rates are >80%. A similar conclusion is drawn when the threshold is set at 1 EU/ml and in both cases the results are not age dependent. When the antibody assay was by RFFIT and the threshold set at 0.5 IU/ml, we might wrongly assume that the vaccine is very efficacious since at the end of 1 year post-vaccination more than 70% of dogs are still seroconverted. However, at that time more than 83% of the dogs were older than 2 years and if one consider the seroconversion rate (almost 60%) of this category of animals at day 0 before vaccination, the vaccination efficacy appear rather marginal after 1 year. If the threshold of seroconversion was set at 1 IU/ml the outcome is better highlighted, since the seroconversion rate was rather low at around 25% at 1 year post-vaccination, compared to the initial 20% rate, before the start of the vaccination protocol.

In conclusion, the simplest way to assess the efficiency of rabies vaccination in dogs is based on the assay of the antibody responses. ELISA is a

straightforward technique which allows a good assessment of the immune responses in experimental conditions, but is rather inconsistent in field conditions and when evaluating residual immunity. RFFIT should still be considered as the reference technique, although it may suffer from the interference of several factors, such as maternal and cross-reacting antibodies and rabies endemicity. This is why the seroconversion threshold of 0.5 IU/ml should be cautiously considered and cannot be extrapolated from one region to another. A higher seroconversion threshold (1 IU/ml) could be more informative in order to efficiently assess the beneficial effect of the rabies vaccination, since it may help to marginalize the impact of interfering factors.

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