

## Comparison of the detection and quantification of rabies antibodies in canine sera

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**ABSTRACT:** Rabies antibodies in canine sera were detected and quantified by methods: virus neutralisation test on mice (VNT), rapid fluorescence focus inhibition test (RFFIT), and fluorescent antibody virus neutralisation (FAVN). The results of rabies antibodies levels in non-vaccinated dogs obtained by all three methods were in correlation. The comparison of rabies antibody titres determined in vaccinated dogs using VNT and FAVN methods showed 86.6% correspondence, while those obtained by RFFIT and FAVN methods corresponded in almost 95% of cases.

**Keywords:** rabies antibodies; detection; RFFIT; FAVN test

Detection and quantification of rabies antibodies is intended in the first place for checking the immunity to rabies or effectiveness of rabies vaccines. Detection and quantification of virus neutralisation rabies antibodies in the serum is based on inhibition of rabies infection *in vivo* in animals or *in vitro* in cell cultures (Atanasiu, 1973; Bourhy and Sureau, 1991). Several suitable procedures have been recommended for determination of titres of virus neutralisation antibodies. The methods most frequently used for quantification of immune response in vaccinated animals after rabies vaccination challenge are serum neutralisation methods carried out on mice and in cell cultures (Smith *et al.*, 1996). WHO recommends *in vivo* virus neutralisation test on mice (VNT) and *in vitro* rapid fluorescence focus inhibition test (RFFIT). The VNT on mice is time demanding and too expensive for routine use in virological laboratories. Recently it has been replaced by sensitive, less expensive and more rapid *in vitro* tests. The RFFIT is highly sensitive and advantageous because of its low time demand. The application of the RFFIT for detection and quantification of rabies antibodies also requires an OIE standard (WHO, 1992). The FAVN (fluorescent antibody virus neutralisation) test was first described by Zalan *et al.* (1979), and later reworked and modified in the AFSSA labora-

tory, Malzéville, Nancy, France. It is simple, rapid, safe and economically sustainable, it allows conduct of many serological examinations needed to check animals, particularly dogs, exported to other countries but also checking the immune status of vaccinated animals (Cliquet *et al.*, 1998, 2000).

## MATERIAL AND METHODS

### Examined sera

Eighty-three sera were chosen for comparison of individual methods for rabies antibodies detection from both non-vaccinated (12 sera) and vaccinated (71 sera) pet dogs. Before examination, they were inactivated by exposure to 56°C for 30 min and stored at –20°C. For comparison of reliability, sensitivity and reproducibility of rabies antibodies detection methods, if the sera were taken from vaccinated dogs we selected those that exhibited titres of rabies antibodies lower than 1.0 IU/cm<sup>3</sup> (International Units/cm<sup>3</sup>), or near the level 0.5 IU per cm<sup>3</sup>, respectively, as detected by the RFFIT. The tests also included titration of reference serum (Copenhagen, Denmark, 30 IU in an ampoule, the dilution of serum containing 0.5 IU/cm<sup>3</sup> antibodies was used for the titration), and the control titration

of reference positive antirabies canine serum and reference negative canine serum (OIE).

### Virus neutralisation test on mice (VNT)

The test was carried out on mice weighing 8–10 g. The mice were inoculated intracerebrally, using 0.03 cm<sup>3</sup> of inoculum, following strictly the WHO recommended method (Atanasiu, 1973). They were challenged with a CVS strain at a dose of 50 MICLD<sub>50</sub>/0.03 cm<sup>3</sup>. The rabies antibody titre was calculated according to Reed and Muench (1938). The comparison of VNT and FAVN methods was performed only with 17 sera (2 sera from the unvaccinated dogs and 15 sera from those vaccinated against rabies), due to high cost of the VNT on mice.

### Rapid fluorescence focus inhibition test (RFFIT)

The test was carried out using the method according to Smith *et al.* (1973), modified by Bourhy and Sureau (1991). All the sera, including the reference one and positive and negative controls, were examined in duplicate on 96-well microtitration plates for cell cultures. The number of fluorescent foci in the cells was expressed as percentage and the titre of rabies antibodies was determined as the dilution of serum, which provided 50% fluorescence reduction. By the RFFIT and parallel by FAVN test 66 sera were examined (10 sera from the unvaccinated dogs and 56 sera from the vaccinated ones).

### FAVN (fluorescent antibodies virus neutralisation) test

The FAVN test is a modified RFFIT method, carried out on 96-well microtitration plates (Cliquet *et al.*, 1998). A stable cell culture BHK-21 (C13) cultivated in a growth medium D-MEM with 10% calf serum was used. After trypsinisation, a cell suspension containing  $4 \times 10^5$  cells/cm<sup>3</sup> was prepared. A standard rabies virus reference strain CVS 11 (Paris) was passaged in a cell culture BHK 21 (C13). The rabies virus or the infectious cellular medium obtained was diluted to obtain 100 TCID<sub>50</sub>/cm<sup>3</sup>. The test also included titration of reference positive and reference negative canine

OIE sera (AFSSA Nancy, France) as well as a WHO reference serum (Copenhagen, Denmark, 30 IU in an ampoule, dilution of serum used for FAVN was 0.5 IU/cm<sup>3</sup>). Every examined serum, including reference WHO serum, reference positive and negative OIE sera, was titrated four times. Triple dilutions 1 : 3, 1 : 9, 1 : 27, 1 : 81, 1 : 243 and 1 : 19 683 were prepared directly on plates. The last dilution was several times higher to record the possibly high antibody titre. Then 50 µl aliquots of CVS 11 virus (100 TCID<sub>50</sub>/cm<sup>3</sup>) were added to the diluted sera in individual wells. After one hour of incubation at 37°C, a cell suspension containing  $4 \times 10^5$  cells per cm<sup>3</sup> was added to each well. After 48 h of incubation at 37°C, the medium was poured off and the plates were washed several times with PBS, fixed for 30 min with 80% acetone at laboratory temperature and dried for approx. 1 hour. After adding a fluorescent conjugate, the plates were incubated again at 37°C for 30 min. The entire surface of each well was evaluated. Results were obtained using the “all or nothing” method. We evaluated first the microtitration plates containing the cell culture tested, titration of CVS, and titration of reference sera and only then the plates with the examined sera. The results were calculated according to Spearman and Kärber (Spearman, 1908; Kärber, 1931).

The results of determination of the immunity level or antibody titre were obtained by comparing ED<sub>50</sub> of the examined serum with ED<sub>50</sub> of the WHO reference serum diluted to 0.5 IU/cm<sup>3</sup>:

- if ED<sub>50</sub> of the examined serum is < ED<sub>50</sub> of the WHO reference serum, then the titre is < 0.5 IU per cm<sup>3</sup>
- if ED<sub>50</sub> of the examined serum is > ED<sub>50</sub> of the WHO reference serum, then the titre is > 0.5 IU per cm<sup>3</sup>

Eighty three sera from vaccinated and unvaccinated dogs were examined by the FAVN test (17 sera were examined by the VNT and 66 by the RFFIT).

## RESULTS AND DISCUSSION

Recently there has been an effort to replace the conventional methods of detection and quantification of rabies antibodies with new methods *in vitro* and to standardise the new tests (WHO, 1992). The FAVN test is a modified method or an adjusted RFFIT, which belongs among the standard WHO methods of determination of antibody titres.

When comparing the serological methods used in diagnostics of infectious diseases it is important to evaluate particularly their sensitivity, specificity, and reproducibility. To prevent errors in results obtained by various methodical procedures it is inevitable to use fully characterised reference standards, reagents, etc. (Briggs *et al.*, 1998).

According to WHO recommendations, the vaccinated animals are protected sufficiently when their level of rabies antibodies equals to or exceeds 0.5 IU/cm<sup>3</sup>. Three different methods, VNT, RFFIT, and FAVN were used in our study to detect rabies virus neutralisation antibodies in selected canine sera. Due to high cost of the VNT on mice, the VNT and FAVN test were compared using only 15 positive and 2 negative sera. They were compared from the point of view of their ability to detect 0.5 IU/cm<sup>3</sup> of antibodies, which is the requirement of WHO and OIE for the minimum level of rabies antibodies capable to ensure immunity status when checking on rabies immunisation. Therefore, the absolute values of rabies antibodies levels obtained by the individual methods were not presented in the results.

Table 1 shows the results obtained by VNT and FAVN test in vaccinated and non-vaccinated dogs.

The two methods were compared using 17 selected sera. The results obtained by the both methods are expressed in IU/cm<sup>3</sup>. The results obtained in non-vaccinated dogs by both methods were in correlation. Comparison of titres of vaccinated dogs showed higher than 86.6% agreement. Two sera with titres close to 0.5 IU/cm<sup>3</sup> were classified as negative by VNT and positive by FAVN test. These particular sera with titres close to 0.5 IU/cm<sup>3</sup> proved that the FAVN test is more sensitive and reliable. The VNT on mice is time demanding, expensive and unpractical for routine use in virological laboratories (Závadová *et al.*, 1996).

Table 2 shows the results of comparison of RFFIT and FAVN test. Analysis included 56 sera from dogs vaccinated against rabies and 10 from the non-vaccinated ones. The comparison of titres obtained by both tests showed 94.7% agreement in vaccinated dogs and 100% in non-vaccinated ones. Three sera with titres close to 0.5 IU/cm<sup>3</sup> were classified as positive by RFFIT and as negative by the FAVN test.

In presented work, we compared the standard diagnostic methods (VNT on mice and RFFIT) with the FAVN test. No significant differences were observed with regard to their sensitivity, spe-

Table 1. The results of titration of rabies antibodies obtained from 2 non-vaccinated and 15 vaccinated dogs using the VNT and FAVN test

	VNT	FAVN	Number of sera	%	Correspondence
Non-vaccinated dogs	–	–	2	100	100
Vaccinated dogs	+	+	10	66.6	86.6
	–	–	3	20.0	
	–	+	2	13.3	13.3
	+	–	0	0.0	

Table 2. The results of titration of rabies antibodies from 10 non-vaccinated and 56 vaccinated dogs by the RFFIT and FAVN test

	RFFIT	FAVN	Number of sera	%	Correspondence
Non-vaccinated dogs	–	–	10	100	100
Vaccinated dogs	+	+	45	80.4	94.7
	–	–	8	14.3	
	–	+	0	0	
	+	–	3	5.3	5.3

cificity and reproducibility. However, there were differences in rapidity of the tests, simplicity and easiness of preparation for them, costs of the reagents and equipment of laboratories. With regard to the preparation and performance of the test (RFFIT takes 24–48 hours) and the respective costs, RFFIT appeared better than FAVN. The RFFIT requires lower volumes of examined serum (0.1 cm<sup>3</sup>) than the FAVN (0.2 cm<sup>3</sup>), lower volume of virus, etc. A disadvantage of the RFFIT is the reading of results and their evaluation. The comparison of tests for detection and quantification of rabies antibodies showed no significant differences between RFFIT and the newer FAVN method, recommended by OIE for examination of animal sera. Both the methods allow identification of non-vaccinated animals with 100% accuracy. However, in certain group of immunosuppressive, poorly reacting vaccinated animals both the methods may provide “false positive and false negative” antibody response in those cases in which only one of them is used (Briggs *et al.*, 1998). Because of this, the titre of rabies antibodies in such animals should be determined by two or three methods. In fact, the level of rabies antibodies, determined by the respective tests, gives us an idea whether or not we should keep the dogs in quarantine or whether the quarantine should last 30 or 120 days. These small deviations in the tests can have considerable economical impact on the owners of dogs who import animals to countries in which rabies does not occur or had been eradicated (Briggs *et al.*, 1998).

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