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# Evaluation of a commercial rabies ELISA as a replacement for serum neutralization assays as part of the pet travel scheme and oral vaccination campaigns of foxes

## *Evaluierung eines kommerziellen Tollwut ELISA als Ersatz für Serumneutralisationstests im Rahmen des Pet Travel Schemes und der oralen Immunisierung von Füchsen*

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**Summary**

EU Regulation 998/2003 requires the serological testing of rabies-vaccinated dogs and cats in approved laboratories using serum neutralization tests prior to movement of pet animals between certain EU member states and before pet animals are imported from unlisted third countries. Serum neutralisation tests are also used for measuring the efficacy of oral rabies vaccination programmes conducted in wild carnivore populations. In this study we evaluated an OIE-listed commercial ELISA as a potential replacement for serum neutralization assays under routine conditions as a diagnostic tool for both the serological testing of dog and cat sera as part of pet travel schemes and for follow-up investigations as part of oral vaccination campaigns. When dog and cat sera were analyzed by ELISA, a sensitivity compared to the standard serological test of 36.9–82.0% and 44.4–88.9%, respectively, was calculated depending on the method used. For fox field samples from oral vaccination areas the sensitivity compared to the Rapid Fluorescent Focus Inhibition Test (RFFIT) was 32.4% (95% CI 24.8–40.0%).
In its present format, the ELISA cannot replace standard serological assays neither in the pet travel scheme nor in follow-up investigations of oral vaccination campaigns. The results obtained resemble those of other rabies ELISAs recently evaluated for the same purpose and may therefore exemplify a general misconception (binding versus neutralization) in rabies serology rather than a failure of this ELISA test per se. Also, problems with technical and legislative issues associated with the serological testing of dog and cat sera for non-commercial movement and related to the outcome of this study are addressed.

**Keywords:** rabies, pet travel scheme, oral rabies vaccination of foxes, RFFIT, FAVN, ELISA, dogs, cats, foxes

**Zusammenfassung**

Die EU Verordnung 998/2003 fordert für das nicht kommerzielle Verbringen von Tollwut-geimpften Hunden und Katzen zwischen bestimmten EU Mitgliedsstaaten sowie bei Import aus nicht gelisteten Drittländern eine serologische Testung mittels Neutralisationstest durch zugelassene Laboratorien. Serumneutralisationstests werden auch für die Überprüfung der Effektivität von oralen Tollwutimmunisierungskampagnen in Wildtierpopulationen verwendet. In der vorliegenden Studie wurde ein OIE-gelisteter kommerzieller Tollwut-ELISA als Alternative zu Serumneutralisationstests sowohl für die serologische Testung von Hunde und Katzenseren im Rahmen des Pet Travel Schemes als auch des Monitoring von oralen Tollwutimmunisierungskampagnen evaluiert. Die Analyse von Hunde- und Katzenseren mittels ELSIA ergab eine Sensitivität gegenüber den serologischen Standardtestverfahren von 44,4–88,9 % in Abhängigkeit der verwendeten Berechnungsmethode. Bei Fuchsseren aus Impfgebieten lag die Sensitivität im Vergleich zum Rapid Fluorescent Focus Inhibition Test (RFFIT) bei

nur 32,4 % (95% KI: 24,8–40,0 %). Im gegenwärtigen Design kann der ELISA herkömmliche serologische Standardverfahren im Rahmen des Pet Travel Schemes sowie für das Monitoring von oralen Tollwutimmunisierungskampagnen nicht ersetzen. Die Ergebnisse gleichen denen eines anderen kommerziellen, für die gleiche Zielstellung evaluierten Tollwut-ELISAs und könnten somit ein allgemeines Missverständnis in der Tollwutserologie (bindende vs. neutralisierende Antikörper) veranschaulichen und nicht ein Fehlschlagen dieses ELISA Tests per se. Darüber hinaus werden technische sowie gesetzgebende Probleme im Zusammenhang mit der serologischen Testung von Hunde und Katzenseren im Rahmen des Pet Travel Schemes und des Ergebnisses dieser Studie diskutiert.

**Schlüsselwörter:** Tollwut, ELISA, Pet Travel Scheme, orale Immunisierung von Füchsen, RFFIT, FAVN, Hunde, Katzen, Füchse

## Introduction

Worldwide, classical rabies is one of the most feared zoonoses. Some countries have either been freed from rabies by controlling the disease in dogs and in wildlife or are historically regarded rabies-free. One possibility of rabies re-introduction is the movement of pet animals incubating the disease. Therefore strict risk mitigating measures have been taken to avoid such a scenario. Lengthy quarantine procedures which are effective but costly and strenuous to pets and their owners have been largely replaced by other schemes. For example, a "Pet Travel Scheme" was introduced in the UK and later adopted in a similar form in the European Union (Fooks et al. 2002; Kennedy, 1998; EC, 2003).

In general, such schemes include identification, vaccination and serological testing prior to movement. Depending on the status of the country of origin, serological testing for anti-rabies antibodies is required in a minimum of 30 days after vaccination, and the test must be taken at least 3 months prior to entry into the European Union in addition to vaccination and the unambiguous identification of the animal using a microchip.

Serological testing in the frame of the EU regulation can only be performed in approved laboratories using either the fluorescent antibody virus neutralization test (FAVN, Cliquet et al., 1998) or the rapid fluorescent focus inhibition test (RFFIT, Smith et al., 1973). Both tests are based on the neutralization of a fixed dose of rabies virus by antibodies present in the tested serum and hence measure virus-neutralizing antibodies (VNA). The tests are sensitive and specific but require specially equipped laboratories and skilled technicians. Since a titre of 0.5 international units per ml (IU/ml) of VNA has been regarded as the minimum protective VNA level in humans (WHO, 1992), this value has also been adopted for animals (Aubert 1992, Briggs and Schweitzer, 2001).

As compared to the neutralization tests, enzyme linked immunosorbant assays (ELISA) are easier to use, cheaper and do not require facilities with a high biosafety level. Several ELISAs have been described for the quantification of rabies antibodies in humans (Kavaklova et al., 1984; Piza et al., 1999; Muhamuda et al., 2007; Welch et al., 2009) and domestic carnivores (Sugiyama et al., 1997; Cliquet et al., 2004; Kasempimolporn et al., 2008; Servat et al., 2007; Zhang et al., 2009). For the detection of rabies antibodies during follow-up investigations of oral rabies vaccination programmes, which are normally performed using various modifications of the RFFIT, different ELISAs have been described or validated (Barton and Campbell, 1988; Cliquet et al., 2000, 2003; Servat et al., 2007). For pet travel movement, a qualitative indirect ELISA (Serelisa™ Rabies Ab mono Indirect, Synbiotics) to measure the level of rabies antibodies in dogs and cats vaccinated against rabies was described by the OIE (2004). Cliquet et al. (2004) found that this test had a relatively low sensitivity. An evaluation performed by different approved laboratories has been published (Servat and Cliquet, 2006). More recently, an indirect ELISA (Platelia™ Rabies II Kit, Bio-Rad), listed by OIE in 2007, was validated (OIE, 2007a; Servat et al., 2007) and its reproducibility evaluated by an inter-laboratory trial (Feyssaguet et al, 2007; Servat et al, 2008). According to the manufacturer's instructions, this ELISA is intended for use in international animal movement and in follow-up investigations of oral rabies vaccination in wildlife.

The aim of our study was to evaluate the OIE listed Platelia™ Rabies II Kit test as a potential replacement for serum neutralization assays, e. g. FAVN and RFFIT, for vaccinated dogs and cats in an approved laboratory under routine conditions. Also, the ELISA needed to be assessed as a diagnostic tool for both the serological testing of dog and cat sera in the context of the pet travel scheme and in follow-up investigation of oral vaccination campaigns. The results of the study are also discussed against the background of current requirements and standards in international legislation and recent international discussions.

## Material and Methods

### Sera

Four panels of sera were selected for testing:

I) 404 serum samples from routine submissions to IDEXX Vet Med Labor, 120 of which were from vaccinated cats and 284 from vaccinated dogs. All sera were submitted in the context of regulation (EC) 998/2003 (movement of pets between countries) and tested using the FAVN within 24 h. Before testing in the FAVN, the sera were stored at 4°C. Thereafter, the samples were stored at –20°C until tested in the ELISA.

II) 44 serologically positive and negative sera (proficiency testing samples from the European Union Reference Laboratory (EU-RL), Agence française de sécurité

**TABLE 1:** *Diagnostic sensitivity and specificity with 95% confidence limits (CI) as calculated with routine dog and cat sera using different calculation methods*

| | dogs | | | | | | cats | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calculation method | A | | B | | C | | A | | B | | C | |
| ELISA\FAVN | pos | neg | pos | neg | pos | neg | pos | neg | pos | neg | pos | neg |
| pos | 182 | 13 | 82 | 0 | 155 | 48 | 88 | 6 | 44 | 2 | 75 | 17 |
| neg | 40 | 49 | 140 | 62 | 67 | 14 | 11 | 15 | 55 | 19 | 24 | 4 |
| sensitivity | 82.0% | | 36.9% | | 69.8% | | 88.9% | | 44.4% | | 75.8% | |
| CI | 76.9–87.0% | | 30.6–43.3% | | 63.8–75.9% | | 82.7–95.1% | | 34.7–54.2% | | 67.3–84.2 | |
| specificity | 79.0% | | 100.0% | | 22.6% | | 71.4% | | 90.5% | | 19.0% | |
| CI | 68.9–89.2% | | 100.0% | | 12.2–33.0% | | 52.1–90.7% | | 77.9–100.0% | | 2.2–35.8% | |

sanitaire des aliments [Afssa], Nancy, of the years 2004–2006) tested by the FAVN.

III) Reference samples from naive or experimentally vaccinated wildlife animals (18 fox sera, 13 raccoon dog sera) repeatedly tested by the RFFIT.

IV) 308 field samples from free living foxes of undefined serological status from two different areas of oral rabies vaccination in Germany from the federal states of Brandenburg (1992–1994) and Rhineland-Palatinate (2006).

All sera were tested both by the serum neutralization assays FAVN and RFFIT, and ELISA, i. e. the commercial "Platelia™ Rabies II Kit".

### Standard reference serological methods

Rabies VNA were measured using the FAVN or the RFFIT essentially as described by Cliquet et al. (1998) or Smith et al. (1973), respectively, adapted as detailed in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2004). Briefly, a constant dose of virus producing approximately 40 infected cell foci ("challenge virus standard"; CVS) was incubated at 37°C and 5% $CO_2$ for one hour with serial dilutions of the respective serum in a microtitre plate. A pre-defined amount of BHK-21 cells was then loaded into the wells of the plate. Along with the serum dilutions, either a calibrated WHO international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Biological Standards and Control, Potters Bar, UK) or an OIE reference serum of dog origin (CRL, Afssa, Nancy) adjusted to 0.5 IU/ml as well as a negative control serum was used on each plate. The plates were incubated for 48 h or 24 h, respectively, at 37°C and 5% $CO_2$. After staining with a fluorescein isothiocyanate (FITC) anti-rabies conjugate (Fujirebio Diagnostics, Malvern, USA), the number of infected cell foci was counted and the respective neutralization titre calculated using the formula of Spearman and Kärber (Kärber, 1931). The VNA titre was defined as the dilution of the test serum showing a 50% reduction of the test virus (50% neutralizing dose, $ND_{50}$). Subsequently, VNA titres were compared to the titre of the reference serum and converted to IU/ml. A titre of =/> 0.5 IU/ml was regarded as positive.

### ELISA (Platelia™ Rabies II ad usum veterinarium)

The "Platelia™ Rabies II Kit ad usum veterinarium" and all reagents for the ELISA were either purchased from or provided by Bio-Rad (Marnes-La-Coquette, France). Three different lots (6K1012, 7E0010, 7E0016) were used. The OIE reference serum of canine origin provided by the EU-RL, Afssa, Nancy, France was used as a positive control (PC) serum. Samples were tested strictly according to the manufacturer's instructions. Briefly, all test serum samples were diluted 1/100 and 100 µl of this dilution were distributed on a microtitre plate coated with rabies glycoprotein G along with PC and negative control (NC) sera. Plates were incubated for 1 h at 37°C. Rabies antibody/glycoprotein G complexes were then detected by addition of 100 µl of POD conjugate. The plates were incubated for 1 h at 37°C, washed and 100 µl TMB chromogen solution were added to each well. The plates were then incubated in the dark for 30 min at room temperature. The colour reaction was stopped with 1 N $H_2SO_4$. Plates were read bichromatically at 450 and 620 nm.

Depending on the purpose of the serological testing, two different assessments were conducted: (i) a quantitative approach intended for individual testing of dog and cat sera in the context of the pet travel scheme or (ii) a qualitative approach for testing of fox and raccoon dog sera in follow-up investigations of oral vaccination campaigns. For the quantitative assessment, two PCs of dog origin were included. One consisted of a 1/100 dilution of a positive serum containing 0.5 IU/ml of VNAs. The second PC had to be prepared as a two-fold serial dilution which allowed to obtain a calibration curve resembling VNA titres as obtained in FAVN of 0.125, 0.25, 0.5, 1, 2 and 4 IU/ml. Subsequently, the quantity of anti-rabies antibodies in the test samples was determined essentially as described by Servat et al. (2007) by comparing the optical density (OD) of the sample to a standard curve constructed with a function of the OD values obtained for each quantification of the PC. For the calculation of Equivalent Units per milliliter (EU/ml) in the quantitative test, three different methods were applied and exclusively used for the samples obtained by routine testing (panel I):

**TABLE 2:** *Diagnostic sensitivity and specificity with 95% confidence limits (CI) as calculated using proficiency test samples (panel II), reference fox and raccoon dog sera (panel III) or field fox samples (panel IV)*

| Group | II | | III | | IV | |
|---|---|---|---|---|---|---|
| ELISA\(FAVN, RFFIT*) | pos | neg | pos | neg | pos | neg |
| pos | **17** | **1** | 26 | 0 | **47** | **0** |
| neg | **9** | **17** | 5 | 3 | **98** | **163** |
| sensitivity | 65.4% | | 83.9% | | 32.4% | |
| CI | 47.1–83.7% | | 70.9–96.8% | | 24.8–40.0% | |
| specificity | 94,44% | | 100.0% | | 100.0% | |
| CI | 83.9–100.0% | | 100.0% | | 100.0% | |

* panel III and IV were tested using RFFIT.

A) according to the manufacturer's instructions as provided with the kit (linear regression)

B) according to instructions obtained from the EU EU-RL for Rabies serology, Afssa, Nancy, France

C) according to instructions obtained from the manufacturer Bio-Rad, Marnes-La-Coquette, France.

For methods B and C, information on the mathematical model used to determine the EU/ml was lacking because either access to the formula was blocked in the spreadsheets provided for the calculations or information was not provided. All three possible figures were used for further comparisons. Quantitative assessment of samples strictly followed the manufacturer's instructions only (method A).

For the qualitative assessment, the PCs were used at predetermined dilutions resembling VNA titres of 0.5 and 4 IU/ml only. Subsequently, the OD of the test sample was compared to that of the PCs. A titre of > 0.5 EU/ml was regarded as positive for both assessments. For any independent run, the manufacturer's validation criteria were strictly followed and test runs repeated if they had failed to meet these criteria.

Panel I was only used for quantitative, and panel IV only for qualitative assessments. All other panels were tested both quantitatively and qualitatively. Values for the diagnostic sensitivity and specificity were calculated relative to the standard serological reference methods FAVN and RFFIT (998/2003 EC) using WinEpiscope 2.0 (Thrusfield et al., 2001).

## Results

### Quantitative and qualitative assessment

In serum samples from routine submissions of vaccinated animals (panel I) 222 dog sera showed a VNA titre equal or above 0.5 IU/ml and 62 had a titre below 0.5 IU/ml using the FAVN/RFFIT. For cat sera 99 of 120 tested were above and 21 below the threshold of 0.5 IU/ml (Tab. 1). Depending on the calculation method (A, B or C), the diagnostic sensitivity of the ELISA based on dog and cat sera varied considerably ranging from 36.9% (95% CI 30.6–43.3%, B) to 82.0% (95% CI 76.9–87.0%,A) for dogs and from 44.4% (95% CI 34.7–54.2%, B) to 88.9% (95% CI 82.7–95.1%, A) for cat sera (Tab. 1).

The diagnostic specificity was highest with method B followed by A and C (Tab. 1). The sensitivity of the test for the dog sera of panel II (proficiency testing) was lower than 65.4% (95% CI 47.1–83.7%; Tab. 2). Using a qualitative assessment, the sensitivity of the ELISA compared to the FAVN was 83.9% (95% CI 70.9–96.8%; Tab. 2) for experimentally obtained fox and raccoon dog sera (panel III), however, when the quantitative approach was applied to panel III, the sensitivity of the ELISA ranged between 68% and 94%, depending on the calculation method (data not shown). Of the 308 fox sera (panel IV) from oral vaccination areas from Germany 145 (47.1%) showed VNA as determined by RFFIT. When analyzed by ELISA, only 47 (15.3%) were classified as positive resulting in a sensitivity compared to the RFFIT of 32.4% (95% CI 24.8–40.0%) for fox field samples from oral vaccination areas (Tab. 2). When the EU/ml and IU/ml values for dog sera of panel I were compared by applying calculation method A, the ELISA titres (EU/ml) were 2 times lower than those obtained in standard serological assays, on average, resulting in a linear regression curve with a slope of 0.52 (Fig. 1A). For cat sera, reference sera and sera from proficiency testing (panel I–III) the ELISA titres (EU/ml) were even 5 times lower than those obtained in FAVN/RFFIT, on average, resulting in linear regression curves with slopes of between 0.21 and 0.25 (Fig. 1C, 1D). Generally, the coefficients of determination ($R^2$) ranged between 0.44 (Fig. 1A) and 0.16 (Fig.1B).

### Performance of the ELISA in titre-groups

According to the titres in the FAVN, the sera of panel I were grouped as follows: group 1 = <0.5 IU/ml (negative sera), group 2 = 0.5 < x < 2.0 IU/ml (positive sera), group 3 = 2.0 < x < 4.0 IU/ml (positive sera), group 4 = > 4.0 IU/ml (positive sera). In general, the highest concordance of results was observed in group 1, but differed in all groups using the various calculation techniques of EU/ml (Fig. 2). Regarding the latter issue, the highest percentages of false negative results were obtained with method B, followed by C and A, and ranged from 4.8% in group 4 to 94.8% in group 2 (Fig. 3).

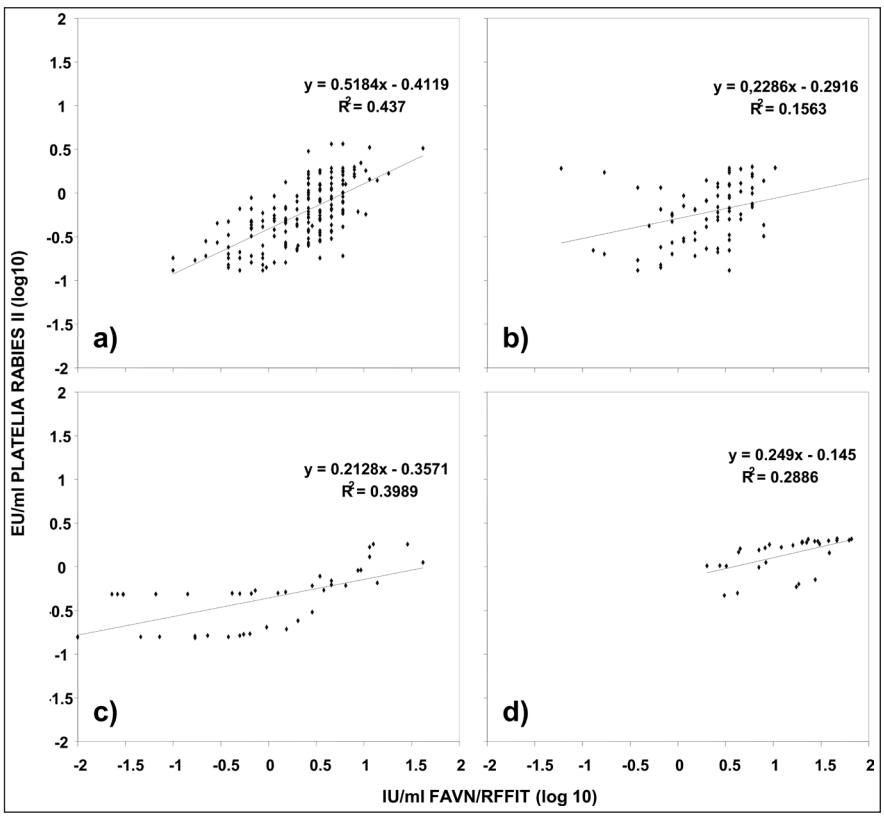


**FIGURE 1:** *Comparison of IU/ml & EU/ml values with dog sera (A: panel I, N = 177), cat sera (B: panel I, N = 79), sera from FAVN proficiency trials (C: panel II, N = 44), and wildlife reference sera (D: panel III, N = 31).*

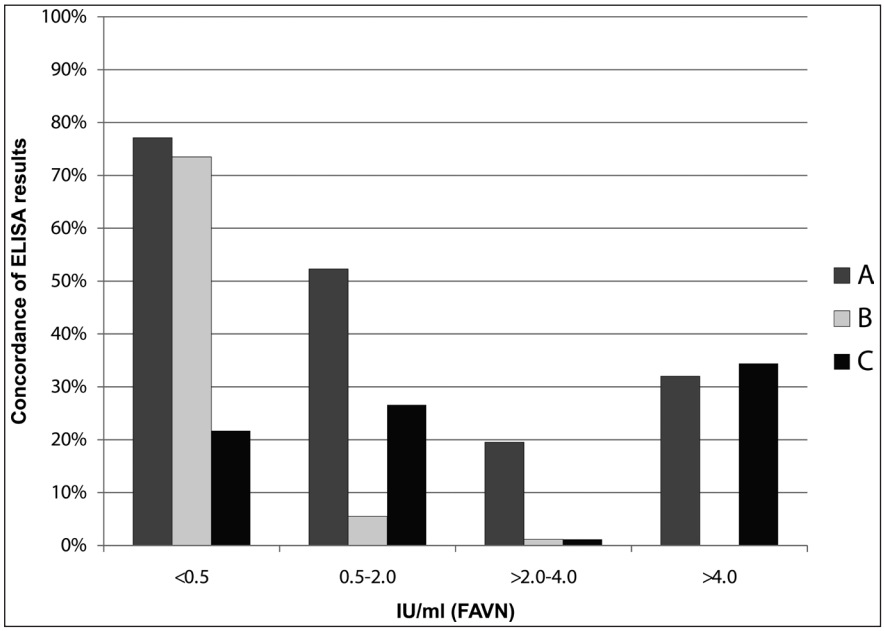


**FIGURE 2:** *Percentage of false negative ELISA results in different titre groups (panel I).*

## Discussion

ELISA-based methods for the measurement of rabies antibodies have been under development for well over two decades. One of the latest products is the Bio-Rad Platelia™ Rabies II. This test is intended to replace the current standard serological reference assays in the pet travel scheme and in the follow-up of oral vaccination campaigns in wildlife. According to the manufacturer's dossier, the test has a diagnostic sensitivity of 80.4–88.9% and a specificity of 98.2–99.2% for dog, cat and fox sera compared to seroneutralization assays (OIE, 2007b). Similar results were also provided in validation studies by other groups (Servat et al., 2007). In our study, we could not confirm these values. Regardless of the origin of samples and the calculation method used, the sensitivity of the ELISA never exceeded 90% in our hands. Many sera which had been shown to contain VNAs reacted negative in the ELISA. Also, the results of the linear regression analyses of the EU/ml and IU/ml values versus the ELISA results for panel II and III (Fig. 1C, 1D) contrast those obtained in other studies (Servat et al., 2007). Actually, based on the calibration curve the EU/ml values are expected to be very similar or even equal to the IU/ml values obtained in standard serological assays resulting in a linear regression function with a slope of nearly 1 and an almost ideal coefficient of determination ($R^2$) close to 1. In our study, however, the EU/ml values were 2 and 5 times lower, on average, resulting in a constant underestimation of antibodies compared to the FAVN /RFFIT. Only 40% of the variation in EU/ml can be explained by the linear regression function (Fig. 1C).

There may be a number of reasons why the ELISA actually failed to meet the expectation of yielding results which can be directly converted into VNA titres, IU/ml or EU/ml values. One main obstacle was the missing standard calculation of EU/ml by the OD values obtained in the ELISA. The description as provided with the kit indicated a linear regression for the OD versus EU/ml value at the time of testing. However, this is not the closest mathematical function of the calibration curve. A logarithmic function, e. g. a data driven approach as described before (Servat et al. 2008) would result in EU/ml values that fit better to the "gold standard" FAVN/RFFIT results. Moreover, there seemed to be no standardization of the calculation method for the quantitative approach for testing of dog and cat sera under routine conditions at the time, as the values for the diagnostic sensitivity and specificity obtained with the calculation methods provided by the EU-RL and the manufacturer varied considerably from those obtained when the manufacturer's instructions were applied (Tab. 1). Any comparison of our data to those obtained in other studies and the technical dossier (Servat et al., 2007; OIE 2007b) is therefore biased. Unfortunately, it was impossible to compare the mathematical models used to determine the EU/ml for the two other methods (B and C) as access to formulas was blocked in the provided Excel® spreadsheets. Nevertheless, our results demonstrate that the three algorithms yielded results which differed considerably (Tab. 1). The use of different test and calculation methods leading to divergent results in the routine testing of dog and cat sera as part of the pet travel scheme would cause considerable confusion among pet owners, approved laboratories and competent authorities. Thus, there is an urgent need to standardize the calculation method using a data driven approach.

Furthermore, it is argued that there may be a certain degree of degradation in some serum samples which may affect the ELISA but not necessarily the FAVN/RFFIT (Morize et al., 2008; Wasniewski and Cliquet, 2008). Degradation of Ig heavy chains can apparently lead to aberrant results. However, such severe negative effects have not been described for ELISAs used to detect other diseases; maybe other characteristics have been implemented to compensate this effect. If this is a result of the binding strength of the secondary detection method (protein A) remains to be clarified. While they need to be addressed and excluded as much as possible in validation and proficiency studies, a certain degree of variability in the quality of sera must be expected under field conditions. Any test must therefore be robust enough to avoid the false classification of results due to serum quality. Also, it has been shown before that the attachment of the G protein to polystyrene microtiter plates can lead to conformational changes that hamper the binding of antibodies (Al Yousif et al. 2000). Purification, concentration and binding of the G-protein may also lead to changes so that some immunoglobulins, perhaps particularly neutralizing antibodies, will no longer bind to some epitopes. This would be another possible explanation for the low concordance observed between ELISA and FAVN/RFFIT.

With respect to testing wildlife samples from the field, the performance of the ELISA was inacceptable.

The test failed to reach the diagnostic sensitivity and specificity for other fox sera calibrated by an in-house ELISA and validated during an inter-laboratory assessment (Cliquet et al. 2000, 2003). In our study, the lack of concordance between the sensitivity of the test for sera obtained from experimentally infected or immunized foxes and raccoon dogs versus field samples (Tab. 2) may be a result of the quality of the field sera, which normally represent transudates from the thoracic cavity. However, such samples can be reliably tested by the RFFIT (Schaarschmidt et al., 2002). One would expect that a binding assay such as the ELISA should be even more sensitive. If the cut-off for the qualitative assessment is reduced, the correlation coefficient in linear regression analyses of the ELISA results versus VNA titres obtained with RFFIT could be improved (data not shown). However, on a population basis, a few false positives and negatives could be neglected if the overall correlation to the expected herd immunity in foxes and/or raccoon dogs after oral vaccination is demonstrated.

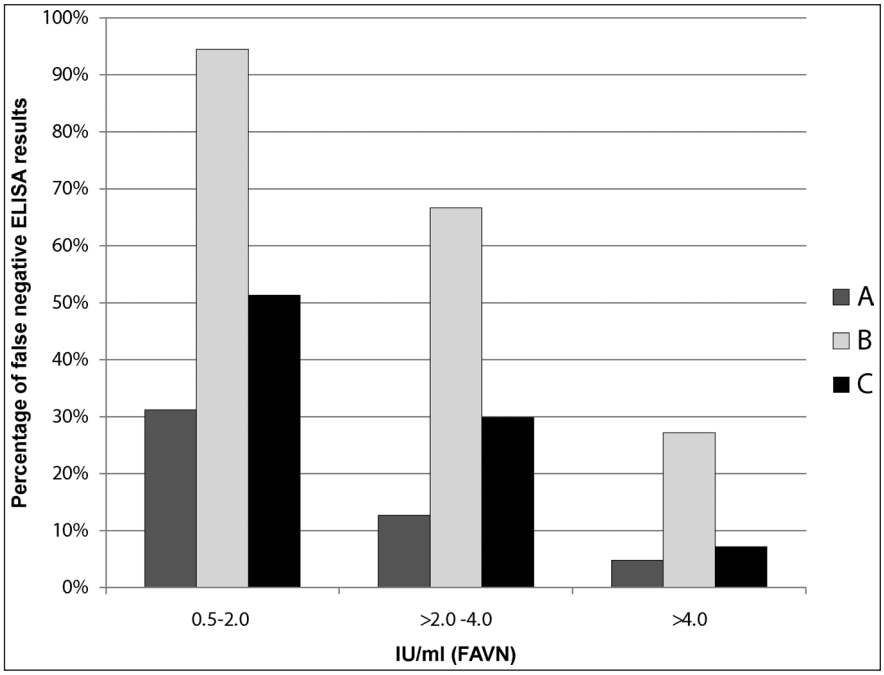


**FIGURE 3:** *Concordance of ELISA versus FAVN results in titre groups (panel I) using different calculation methods (A, B, C).*

If it is intended to replace a neutralization assay by an ELISA and by translating OD values obtained with a test that measures antibodies directed against a variety of epitopes including non-neutralizing sites into neutralizing antibody titres or even more complex measures such as IU/ml or EU/ml, there are inherent limitations of the ELISA technique. The Bio-Rad Platelia™ Rabies II like the Serelisa™ Rabies Ab mono Indirect (Synbiotics) is based on the detection of binding antibodies to the rabies glycoprotein and was as such adapted to the standard serological reference methods (FAVN/RFFIT) using the 0.5 IU/ml threshold (Cliquet et al., 2004; Servat and Cliquet, 2006; Servat et al., 2007). The inherent limitation of ELISA methodology versus a functional test like virus neutralization assays is that: (i) not all neutralizing antibodies act through binding to protein moieties, in this instance glycoprotein G, offered in its native form, and (ii) not all antibodies that bind to G in its native form actually neutralize virus. In combination with the chosen cut-offs in the respective assays this is the most plausible explanation for the discordant results obtained both in ELISA and standard serological assays (FAVN/RFFIT) using the same samples. In general, if a high titre is found by virus neutralization tests (VNT), a high level of reactivity is also confirmed by the ELISA technique – but there may be substantial variation in individual samples. In panel I most of the discordant results were found in titre groups 2 to 4 (Fig. 2) with the highest percentages of false negative results obtained in group 2 (Fig. 3). Thus, the results showed that depending on the calculation method there was a certain test agreement between the ELISA and VNTs, however, it does not provide an acceptable solution for the determination of individual VNA titres using an ELISA. Servat and Cliquet (2006) have tried to solve this problem by applying a ROC analysis to define the optimal cut-off. However, it remains elusive why then the ELISA performance in this study is not sufficiently accurate.

The current EU (EC 998/2003) and OIE regulations (OIE, 2009) for the movement of pet animals from certain countries require a titre of at least 0.5 IU/ml of VNA for each individual as an approximation for protection by prior vaccination. Since binding assays do not necessarily show the correct neutralizing activity of sera, the ELISA per se is not the correct test to be used as a standard method. If individual VNAs are required serum neutralizing assays are a prerequisite and any ELISA can only be used as a screening tool regardless of its design. It is therefore questionable why the OIE has validated this test as fit for purpose, i. e. for the determination of immune status post-vaccination in individual dogs or cats (for regulation of international movement or trade), and in fox populations (for monitoring wildlife vaccination programmes) (OIE, 2007a).

If used as a screening tool, the cut-off value needs to be adjusted to a level where false positive results are avoided and negative and borderline samples up to a titre of 1.0 EU/ml would need to be confirmed by a serum neutralizing assay. From a practical point of view, such a double test strategy could result in more costs for some pet owners while the laboratories would need to maintain the equipment for both tests.

Another theoretical scenario is a change in the current legislation in the sense that serological tests are only used to check for seroconversion as a result of successful vaccination. Such an approach would fit better to the efficacy trials to be conducted as part of the registration process of inactivated rabies vaccines for pets where protection due to immunization is measured as survivorship after challenge infection. Moreover, risk analyses have shown that a serum test only adds minimal safety if an adequate waiting period is guaranteed (EFSA, 2006). In this case, this would also suit the diagnostic concept of an ELISA to detect binding antibodies rather than VNAs. A simple yes or no

decision would be sufficient to check if an animal has developed rabies-specific antibodies or not indicating the animal was vaccinated or not. Thus, a serum test of any kind including ELISAs may be beneficial especially for the compliance to the regulations.

As long as this problem is not solved yet, any attempts to replace standard serological assays measuring VNAs by ELISA for pet travel in the frame of EU regulation 998/2003 or by OIE regulation are not scientifically justified.

## Conclusion

The Bio-Rad Platelia™ Rabies II in its present specification is not an alternative to replace standard serological assays for pet travel and for follow up investigations of oral vaccination campaigns. The problems associated with this ELISA resemble those with the recently evaluated Serelisa™ Rabies Ab mono Indirect (Servat and Cliquet, 2006) and may therefore exemplify a general misconception in rabies serology, consisting of a combination of complex technical and legislative issues. It seems that the threshold of 0.5 IU/ml as an approximation of successful vaccination for animals needs to be reconsidered. Especially with respect to fox sera from the field, the performance of the ELISA was poor. It can therefore not be recommended for this application in its current design. There is no doubt that rabies ELISAs per se have a great potential for serological testing. However, in every situation the aim of the test must be defined first and then the appropriate cut-offs need to be determined separately to make them fit-for-purpose.

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## References

**Al-Yousif Y, Al-Majhdi F, Chard-Bergstrom C, Anderson J, Kapil S (2000):** Development, characterization and diagnostic applications of monoclonal antibodies against bovine rotavirus. Clin Diagn Lab Immunol 7: 288–292.

**Anders G, Vakil HB (2007):** ELISA assays for rabies antibodies? Hum Vaccines 3: 156.

**Aubert MF (1992):** Practical significance of rabies antibodies in cats and dogs. Rev Sci Tech 11: 735–760.

**Briggs DJ, Schweitzer K (2001):** Importation of dogs and cats to rabies-free areas of the world. Vet Clin North Am Small Anim Pract 31: 573–583.

**Barton LD, Campbell JB (1988):** Measurement of rabies-specific antibodies in carnivores by an enzyme-linked immunosorbent assay. J Wildl Dis 24: 246–258.

**Cliquet F, Aubert MFA, Ságnet L (1998):** Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods 212: 79–87.

**Cliquet F, Sagné L, Schereffer JL, Aubert MFA (2000):** ELISA test for rabies antibody titration in orally vaccinated foxes sampled in the fields. Vaccine 18: 3272–3279.

**Cliquet F, Müller T, Mutinelli F, Geronutti S, Brochier B, Selhorst T, Schereffer JL, Krafft N, Burow J, Schameitat A, Schlüter H, Aubert M (2003):** Standardisation and establishment of a rabies ELISA test in European laboratories for assessing the efficacy of oral fox vaccination campaigns. Vaccine 21: 2986–2993.

**Cliquet F, McElhinney LM, Servat A, Boucher JM, Lowings JP, Goddard T, Mansfield KL, Fooks AR (2004):** Development of a qualitative indirect ELISA for the measurement of rabies virus-specific antibodies from vaccinated dogs and cats. J Virol Methods 117: 1–8.

**EC/European Commission (2003):** Regulation (EC) No 998/2003 of the European Parliament and of the Council of 26 May 2003 on the animal health requirements applicable to the non-commercial movement of pet animals and amending Council Directive 92/65/EEC.

**EFSA/European Food Safety Agency (2006):** Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) on a request from the Commission regarding an assessment of the risk of rabies introduction into the UK, Ireland, Sweden, Malta, as a consequence of abandoning the serological test measuring protective antibodies to rabies. Adopted 11 December 2006, http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620772660.htm

**Feyssaguet M, Dacheux L, Audry L, Compoint A, Morize JL, Blanchard I, Bourhy H (2007):** Multicenter comparative study of a new ELISA, PLATELIATM RABIES II, for detection and titration of anti-rabies glycoprotein antibodies and comparison with the rapid fluorescent focus inhibition test (RFFIT) on human samples from vaccinated and non-vaccinated people. Vaccine 25: 2244–2251.

**Fooks AR, McElhinney L, Brookes SM, Johnson N, Keene V, Parsons G, Soldan, A (2002):** Rabies antibody testing and the UK Pet Travel Scheme. Vet Rec 150: 428–430.

**Kärber G (1931):** Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. Arch Exp Pathol Pharmakol 162: 480–483.

**Kavaklova L, Eskenazy M, Gancheva T, Vacheva V (1984):** Enzyme-linked immunosorbent assay for quantification of rabies antibodies in human sera. Acta Virol 28: 422–427.

**Kasempimolporn S, Sichanasai B, Saengseesom W, Puempumpanich S, Sitprija V (2008):** Stray dogs in Bangkok, Thailand: rabies virus infection and rabies antibody prevalence. Dev Biol 131: 137–143.

**Kennedy, J (1998):** Quarantine and rabies: a reappraisal. Report by the Advisory Group on Quarantine on the Right Hon Nick Brown MP, Minister of Agriculture, Fisheries and Food. MAFF Publications, London.

**Morize JL, Bilheude JM, Sonigo P (2008):** Exploration of variations encountered with Platelia Rabies 2 during the proficiency test organised by Afssa Nancy Laboratory. Technical report by Bio-Rad Laboratories, Marnes-la Coquette, France: 1–8.

**Muhamuda K, Madhusudana SN, Ravi V (2007):** Development and evaluation of a competitive ELISA for estimation of rabies neutralizing antibodies after post-exposure rabies vaccination in humans. Int J Infect Dis 11: 441–445.

**OIE/Office Internationale des Epizooties (2004):** Manual of diagnostic test and vaccines for terrestrial animals. OIE, Paris, France.

**OIE/Office Internationale des Epizooties (2007a):** Resolution o. XXIX, Register of Diagnostic Tests Validated and Certified by the OIE; http://www.oie.int/vcda/eng/Registre/OIE_GS2007_RESOLUTION%20No%20XXIX_eng.pdf, accessed 2010-01-19.

**OIE/Office Internationale des Epizooties (2007b):** OIE Procedure for Validation and Certification of Diagnostic Assays, Abstract sheet: Platelia Rabies II; http://www.oie.int/vcda/eng/Registre/Abstract%20sheet_OIE%20Register_PlateliaRabiesII_v1.pdf, accessed 2010-01-19.

**OIE/Office Internationale des Epizooties (2008):** Rabies. In: Manual of diagnostic tests and vaccines for terrestrial animals. Online edition, http://www.oie.int/eng/normes/mmanual/A_summry.htm. OIE, Paris.

**OIE/Office Internationale des Epizooties (2009):** Rabies. In: Terrestrial Animal Health Code. Online edition, http://www.oie.int/eng/normes/mcode/en_sommaire.htm. OIE, Paris.

**Piza AS, Santos JL, Chaves LB, Zanetti CR (1999):** An ELISA suitable for the detection of rabies virus antibodies in serum samples from human vaccinated with either cell-culture vaccine or suckling-mouse-brain vaccine. Rev Inst Med Trop Sao Paulo 41: 39–43.

**Schaarschmidt U, Müller T, Albert G, Muluneh A, Cox J, Selhorst T, Schlüter H (2002):** Erfahrungen mit der Begleitdiagnostik zur oralen Immunisierung der Füchse in Sachsen unter besonderer Berücksichtigung einer standardisierten Tollwutserologie. Dtsch Tierarztl Wochenschr 109: 219–225.

**Servat A, Cliquet F (2006):** Collaborative study to evaluate a new ELISA test to monitor the effectiveness of rabies vaccination in domestic carnivores. Virus Res 120: 17–27.

**Servat A, Feyssaguet M, Blanchard I, Morize JL, Schereffer JL, Boue F, Cliquet F (2007):** A quantitative indirect ELISA to monitor the effectiveness of rabies vaccination in domestic and wild carnivores. J Immunol Methods 318: 1–10.

**Servat A, Labadie A, Hamen A, Boue F, Cliquet F (2008):** Interlaboratory trial to evaluate the reproducibility of a new ELISA to detect rabies antibodies in vaccinated domestic and wild carnivores. Biologicals 36: 19–26.

**Smith JS, Yager PA, Baer GM (1973):** A rapid reproducible test for determining rabies neutralizing antibody. Bull World Health Organ 48: 535–541.

**Sugiyama M, Yoshiki R, Tatsuno Y, Hiraga S, Itoh O, Gamoh K, Minamoto N (1997):** A new competitive enzyme-linked immunosorbent assay demonstrates adequate immune levels to rabies virus in compulsorily vaccinated Japanese domestic dogs. Clin Diagn Lab Immunol 4: 727–730.

**Thrusfield M, Ortega C, de Blas I, Noordhuizen JP, Frankena K (2001):** WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. Vet Rec 148: 567–572.

**Wasniewski M, Cliquet F (2008):** Technical report on several studies perfomed by Afssa Nancy laboratory on the Platelia Rabies II kit designed by Biorad. Technical report by Afssa, Nancy, France: 1–23.

**Welch RJ, Anderson BL, Litwin CM (2009):** An evaluation of two commercially available ELISAs and one in-house reference laboratory ELISA for the determination of human anti-rabies virus antibodies. J Med Microbiol 58: 806–810.

**WHO/World Health Organisation (1992):** WHO expert committee on rabies 1992, 8th report. WHO technical report series n°824, 84p.

**Zhang S, Liu Y, Zhang F, Hu R (2009):** Competitive ELISA using a rabies glycoprotein-transformed cell line to semi-quantify rabies neutralizing-related antibodies in dogs. Vaccine 5: 2108–2113.

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