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

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
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 Ireland followed by 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 immunosorbent 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

<table>
<thead>
<tr>
<th>Calculation method</th>
<th>dogs</th>
<th></th>
<th>cats</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pos</td>
<td>neg</td>
<td>pos</td>
<td>neg</td>
</tr>
<tr>
<td>ELISA/FAVN pos</td>
<td>182</td>
<td>13</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>neg</td>
<td>40</td>
<td>49</td>
<td>140</td>
<td>62</td>
</tr>
<tr>
<td>sensitivity</td>
<td>82.0%</td>
<td>36.9%</td>
<td>69.8%</td>
<td>44.4%</td>
</tr>
<tr>
<td>CI</td>
<td>76.9–87.0%</td>
<td>30.6–43.3%</td>
<td>63.8–75.9%</td>
<td>82.7–95.1%</td>
</tr>
<tr>
<td>specificity</td>
<td>79.0%</td>
<td>100.0%</td>
<td>74.6%</td>
<td>22.6%</td>
</tr>
<tr>
<td>CI</td>
<td>68.9–89.2%</td>
<td>100.0%</td>
<td>72.2–100.0%</td>
<td>12.2–33.0%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Group</th>
<th>ELISA/FAVN</th>
<th>FAVN</th>
<th>RFFIT*</th>
<th>pos</th>
<th>neg</th>
<th>pos</th>
<th>neg</th>
<th>pos</th>
<th>neg</th>
<th>pos</th>
<th>neg</th>
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<tr>
<td>pos</td>
<td>17</td>
<td>1</td>
<td>26</td>
<td>0</td>
<td>47</td>
<td>0</td>
<td>98</td>
<td>163</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>9</td>
<td>17</td>
<td>5</td>
<td>3</td>
<td>98</td>
<td>163</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sensitivity</td>
<td>65.4%</td>
<td>83.9%</td>
<td>32.4%</td>
<td>47.1–83.7%</td>
<td>70.9–96.8%</td>
<td>24.8–40.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>specificity</td>
<td>94.44%</td>
<td>100.0%</td>
<td>100.0%</td>
<td>83.9–100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

* 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 (R²) 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).](image-url)
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 two decades. One of the latest products is the Bio-Rad antibodies have been under development for well over ELISA-based methods for the measurement of rabies Discussion linear regression function (Fig. 1C). 40% of the variation in EU/ml can be explained by the tion of antibodies compared to the FAVN /RFFIT. Only lower, on average, resulting in a constant underestima- tion function with a slope of nearly 1 and an almost standard serological assays resulting in a linear regres- sion curve the EU/ml values are expected to be very studies (Servat et al., 2007). Actually, based on the cali- bration 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 (R²) close to 1. In our study, however, the EU/ml values were 2 and 5 times lower, on average, resulting in a constant underestima- tion 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 func- tion, 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 sen- sitivity 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 compari- son of our data to those obtained in other studies and the technical dos- sier (Servat et al., 2007; OIE 2007b) is therefore biased. Unfortunately, it was impossible to compare the mathemat- ical 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 dif- fered 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 confu- sion 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 nega- tive 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 (AlYousif et al. 2000). Purifi- cation, 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 unacceptable.
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.

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 (Symbiotics) 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 ELISA (98/288/EC) 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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The authors declare that they have no competing interests.

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