An evaluation of two commercially available ELISAs and one in-house reference laboratory ELISA for the determination of human anti-rabies virus antibodies

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The envelope glycoprotein G of rabies virus in vaccines induces the production of neutralizing antibodies important in the protection against the disease. The measurement of anti-envelope glycoprotein antibodies is a good predictor of the degree of humoral immunity in people during anti-rabies treatment or after vaccination. Several assays exist for the serological determination of antibody protection against rabies virus infection. Antibody neutralization by the rapid fluorescent focus inhibition test (RFFIT) or the fluorescent antibody virus neutralization (FAVN) test is currently the gold standard. Performance of the highly complex RFFIT and FAVN tests, however, requires specialized reference laboratories with expertise with this assay. Although not widely used, ELISA test kits are available and may be an additional option for testing that is more accessible. The aim of the present study was to evaluate available ELISA assays for the determination of anti-rabies antibodies. We compared the Bio-Rad Platelia Rabies II ELISA, DRG Rabies Virus IgG Ab ELISA and Focus Diagnostics Rabies Antibody Detection by ELISA to RFFIT. Bland–Altman plots comparing the Bio-Rad Platelia assay and the Focus Diagnostics assay to RFFIT showed a low degree of variability between the ELISA assays and RFFIT results except in samples with high RFFIT values. The agreement, sensitivity and specificity of Bio-Rad Platelia Rabies II ELISA when compared to RFFIT were 95.1 %, 94.1 % and 95.8 %, respectively. The DRG Rabies assay compared to RFFIT had an agreement of 77.7 %, a sensitivity of 86.7 % and a specificity of 69.4 %. The agreement, sensitivity and specificity of Focus Diagnostics Rabies Detection by ELISA when compared to RFFIT were 82.2 %, 91.7 % and 73.0 %, respectively. Overall, the Bio-Rad Platelia assay showed higher accuracy and specificity than either the DRG or Focus assays. All of these ELISAs, however, measure all antibody types and do not discriminate the neutralizing antibodies as measured by functional assays (RFFIT and FAVN) and cannot be relied upon to predict the neutralizing activity of the sera. The results of this study offer insight into the availability of alternative, less-complex methods to monitor rabies antibody titres in at-risk individuals following vaccination.

INTRODUCTION

Rabies is a well-known viral infection that produces consistently fatal encephalitis in most mammals. Humans contract rabies most commonly from bites by animals infected with rabies (Favoretto et al., 2006; Knobel et al., 2005). Of the rabies cases observed worldwide, dogs account for 54 %, terrestrial wildlife (skunks, raccoons, foxes, cats, etc.) for 42 %, and bats 4 % (Krebs et al., 2005; Mandell et al., 2005; Meslin et al., 1994). However, in countries where canine rabies has not been adequately controlled (many Asian, African and Latin American countries), dogs account for 90 % or more of rabies cases (Fishbein & Robinson, 1993). Although bats account for a small percentage of rabies in animals, they have become the most significant source of human rabies infection in the USA (CDC, 2006). Aggressive vaccination programmes in the USA have nearly eliminated rabies in the domestic population, leaving the majority of rabies

Abbreviations: CI, confidence interval; FAVN, fluorescent antibody virus neutralization; RFFIT, fluorescent focus inhibition test; WHO, World Health Organization.

A supplementary table showing raw data for the test methods is available with the online version of this paper.
cases originating in the wild animal population (CDC, 1999, 2006; Krebs et al., 2005).

Because rabies is an invariably fatal illness, individuals at a high risk of exposure to rabies (such as veterinarians, laboratory workers and others at risk of contact with rabid domestic or wild animals) should undergo post-exposure prophylaxis with the rabies vaccine (Krause et al., 2005; Wilde et al., 2003). Rabies vaccine efficacy needs to be monitored for these ‘at-risk individuals’ to ensure protection against infection. Current World Health Organization (WHO) guidelines suggest antibody levels be measured every 6 months in individuals who maintain a continuous risk of exposure and every 2 years for those who maintain frequent exposure to high-risk environments (Murray & Arguin, 2000).

Currently, the reference method accepted by the WHO for monitoring individuals for seroconversion against rabies is seroneutralization, specifically the rapid fluorescent focus inhibition test (RFFIT) or fluorescent antibody virus neutralization (FAVN) test (WHO, 2005). These highly complex measurements of in vitro virus-neutralizing antibodies are difficult and testing availability is limited within the USA. This limited availability has created the need to develop a more rapid and routine methodology for detection of anti-rabies virus specific antibodies. This study compares two commercially available ELISA tests and an established in-house reference laboratory ELISA.

**METHODS**

**Human sera.** A total of 94 human serum samples were tested for anti-rabies antibodies. The samples were divided into two groups.

Group I included 70 serum samples that were submitted to the clinical laboratory for rabies antibody testing. All 70 samples were tested using both commercial ELISA tests, the in-house reference laboratory ELISA and by RFFIT.

Group II included 24 samples from healthy individuals that specifically had no history of rabies vaccination. These samples were tested using both ELISA kits, the in-house reference laboratory ELISA and by RFFIT. Due to low sample volumes, only discrepant results between RFFIT and the Bio-Rad Platelia Rabies II ELISA were repeated to ensure reproducibility.

The procedures followed were in accordance with the ethical standards established by the University of Utah and with the Helsinki Declaration of 1975. All patient samples included in this study were de-identified according to the University of Utah Health Information Portability and Accountability Act patient confidentiality guidelines. Specimens were stored at –20 °C until testing commenced and were then stored at 2–8 °C while all evaluations were performed. All discrepant samples were repeated on each respective assay to ensure reproducibility.

**Commercial ELISAs.** The three ELISA kits included the Bio-Rad Platelia Rabies II kit, DRG Rabies Virus IgG Ab ELISA (DRG International) and Focus Diagnostics in-house developed Rabies Antibody Detection by ELISA.

For the Bio-Rad assay, serum samples were tested according to manufacturer’s specifications, which utilize a glycoprotein antigen (PV strain of rabies and extracted as previously described by Feyssaguet et al., 2007) and peroxidase-labelled protein-A conjugate (Atanasiu & Perrin, 1979; Feyssaguet et al., 2007; Perrin et al., 1986).

Results were expressed in equivalent units (EU) ml⁻¹, which correlates with international units (IU) ml⁻¹. A value greater than or equal to 0.50 EU ml⁻¹ represents aeroconverted antibody level with an equivocal range (questionable level of antibodies for evidence of seroconversion) from 0.125 to 0.500 EU ml⁻¹.

The DRG assay was run according to the manufacturer’s protocol, which uses whole-inactivated virus antigen and horseradish peroxidase-conjugated anti-species antibodies. A cut-off value is calculated by adding 0.200 to the A₄₅₀ of the negative control. A sample with an absorbance higher than the cut-off value suggests seroconversion.

Samples were sent to Focus Diagnostics for antibody testing by their in-house assay for antibodies against purified viral envelope glycoprotein. Results were expressed in IU ml⁻¹. A value greater than or equal to 0.50 IU ml⁻¹ represents aeroconverted antibody level with an equivocal range from 0.30 to 0.50 IU ml⁻¹.

**RFFIT.** All samples from groups I and II were sent to Kansas State University (Manhattan, Kansas, USA) for RFFIT testing. Samples were titrated out and compared to WHO standards with a known antibody concentration to convert the titres into IU ml⁻¹, with a value greater than or equal to 0.50 IU ml⁻¹ considered aeroconverted antibody level.

**Statistical analyses.** The data were analysed with 95% confidence intervals (CI) using a two-way contingency table analysis with a Yates-corrected chi-square test (Fleiss, 1981). The agreement, clinical sensitivity and clinical specificity were determined for the Bio-Rad, DRG and Focus Diagnostics ELISA assays by using results from RFFIT as the reference. Analysis of the data was also performed comparing the Focus and DRG ELISA assays with the Bio-Rad ELISA assay. Equivocal results, as determined by each individual manufacturer, were not included in the calculations.

Linear regression analysis and difference against the mean analysis was performed (Bland & Altman, 1995). Linear regression was done comparing the Bio-Rad and Focus assay against the RFFIT as well as the Focus assay against the Bio-Rad assay. Bland–Altman plots were generated comparing the Bio-Rad and Focus assay against the RFFIT as well as the Focus assay against the Bio-Rad assay.

**RESULTS AND DISCUSSION**

**Comparison of the Bio-Rad Platelia Rabies II ELISA and the RFFIT**

According to the WHO, an antibody level of 0.50 IU ml⁻¹ or greater indicates seroconversion following vaccination (WHO, 2005). When the 70 samples from group I (vaccinated individuals) were tested by RFFIT, 60% (42/70) had antibody concentrations equal to or greater than 0.50 IU ml⁻¹ with 33% (14/42) of those having concentrations between 0.50 and 1.00 IU ml⁻¹. When the 24 samples from the unvaccinated group (group II) were tested by RFFIT, 12.5% (3/24) of the samples had antibody concentrations higher than 0.50 IU ml⁻¹ with 33% (1/3) of those having concentrations between 0.50 and 1.00 IU ml⁻¹. See Supplementary Table S1 in JMM Online.

The agreement, sensitivity and specificity of the Bio-Rad Platelia Rabies II ELISA compared to the RFFIT were...
95.1% (CI 88.1–98.1%), 94.1% (CI 85.7–97.7%) and 95.8% (CI 89.9–98.4%), respectively (Table 1). Four (4/82) total discrepant samples were obtained. Two out of the 34 samples that tested positive by the RFFIT were negative by the Bio-Rad Platelia with 50% (1/2) having RFFIT antibody concentrations between 0.50 and 1.00 IU ml\(^{-1}\), and two out of the 48 samples that tested negative by RFFIT were positive by the Bio-Rad Platelia. All four discrepant samples were from group I. A linear regression analysis on all 94 serum samples (including all equivocal results) was performed to compare the Bio-Rad Platelia to the RFFIT (Fig. 1). The analysis produced a regression coefficient of \(R^2=0.6852\) (\(P<0.0001\)) and a slope of 0.1515.

To assess whether the difference between the Bio-Rad and RFFIT methods was related to the magnitude of each measurement, a Bland–Altman plot was generated to compare the Bio-Rad assay to RFFIT (Fig. 2). The high \(R^2\) of 0.9568 (\(P<0.0001\)) indicated a low degree of variability between the Bio-Rad and RFFIT results. The difference between the Bio-Rad and RFFIT results remained close to 0 for samples with a mean result under 5 but showed a negative correlation overall (slope = −1.4462) as a result of a large difference between the Bio-Rad and RFFIT results when the samples had high RFFIT values.

Table 1. Summary of results comparing the Bio-Rad Platelia ELISA, DRG Rabies Virus IgG Ab ELISA and Focus Diagnostics Rabies Detection by ELISA to the RFFIT

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<th>RFFIT results</th>
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<td>Positive</td>
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<td>Bio-Rad Platelia ELISA results*</td>
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<tr>
<td>Positive</td>
<td>32</td>
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<td>Negative</td>
<td>2</td>
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<td>DRG Rabies Virus IgG Ab ELISA†</td>
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<tr>
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<td>39</td>
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<tr>
<td>Negative</td>
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<td>Focus Diagnostics Rabies Detection by ELISA‡</td>
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<tr>
<td>Positive</td>
<td>33</td>
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*The Bio-Rad Platelia Rabies II compared to the RFFIT resulted in an agreement of 95.1% (CI 88.1–98.1%), a sensitivity of 94.1% (CI 85.7–97.7%) and a specificity of 95.8% (CI 89.9–98.4%).
†The DRG Rabies Virus IgG Ab ELISA compared to RFFIT resulted in an agreement of 77.7% (CI 68.6–83.8%), a sensitivity of 86.7% (CI 77.2–93.1%) and a specificity of 69.4% (CI 60.7–75.3%).
‡The Focus Diagnostics Rabies Detection by ELISA compared to RFFIT resulted in an agreement of 82.2% (CI 82.4–87.4%), a sensitivity of 91.7% (CI 81.8–96.9%) and a specificity of 73.0% (CI 63.3–78.1%).

Comparison of the DRG Rabies Virus IgG Ab ELISA and the RFFIT

When the DRG Rabies Virus IgG Ab ELISA was compared to the RFFIT, the agreement, sensitivity and specificity were 77.7% (CI 68.6–83.8%), 86.7% (CI 77.2–93.1%) and 69.4% (CI 60.7–75.3%), respectively (Table 1). Twenty-one (21/94) total discrepant samples were observed. Six out of the 45 samples that tested positive by RFFIT were negative by the DRG assay with 66% (4/6) having RFFIT antibody concentrations between 0.50 and 1.00 IU ml\(^{-1}\), and 15 out of the 49 samples negative by RFFIT were positive by the DRG assay. All six of the false-negative samples were from group I. Of the 15 false-positive samples, eight were from group I and seven were from group II.
Comparison of Focus Diagnostics Rabies Antibody Detection by ELISA and the RFFIT

The Focus Diagnostics Rabies Antibody Detection by ELISA was compared to the RFFIT. An agreement, sensitivity and specificity of 82.2 % (CI 72.4–87.4 %), 91.7 % (CI 81.8–96.9 %) and 73.0 % (CI 63.3–78.1 %), respectively, was observed (Table 1). Thirteen (13/73) total discrepant samples were observed. Three out of the 36 samples that tested positive by RFFIT were negative by the Focus assay with 66 % (2/3) having RFFIT antibody concentrations between 0.50 and 1.00 IU ml\(^{-1}\), and 10 out of the 37 samples negative by the RFFIT were positive by the Focus assay. All of the false-negative samples were from group I. Of the ten false-positive samples, five were from group I and five were from group II. A linear regression analysis on all 94 serum samples (including all equivocal results) was performed to compare the Focus assay to the RFFIT (figure not shown). The analysis produced a regression coefficient of \( R^2 = 0.6853 \) (\( P < 0.0001 \)). A Bland–Altman plot was generated comparing the Focus assay with the RFFIT (Fig. 2). This analysis produced a plot with an \( R^2 = 0.1603 \) (\( P < 0.0001 \)) and a slope of \(-0.2894\), indicating high variability between the tests.

In general, the DRG assay and the Focus assay were less sensitive than the Bio-Rad Platelia when compared to the RFFIT. It is important to note that the majority of false-negative results were observed in samples with low IU ml\(^{-1}\) values. In this and other studies, samples with moderate or high RFFIT values showed few if any discrepancies with corresponding ELISA antibody concentrations (Briggs et al., 1998). One recent study showed a strong correlation claiming a sensitivity and specificity of 98.96 % and 99.35 %, respectively (Feyssaguet et al., 2007).

The Bio-Rad Platelia showed higher accuracy and specificity than either the DRG or Focus assays. The objective in introducing any ELISA test for rabies compared with a virus-neutralization test is to reduce the number of false-positive results. Therefore, specificity is more important.

Comparison of the DRG Rabies Virus IgG Ab ELISA and Focus Diagnostics Rabies Antibody Detection by ELISA to the Bio-Rad Platelia assay

The DRG assay had an agreement, sensitivity and specificity compared to the Bio-Rad Platelia of 83.1 % (CI 75.3–85.1 %), 97.1 % (CI 87.8–99.5 %) and 72.9 % (CI 66.1–74.6 %), respectively. One false-negative (1/35) and 13 false-positive (13/48) results were observed with the false-negative having a Bio-Rad Platelia antibody concentration between 0.50 and 1.00 EU ml\(^{-1}\).

The agreement, sensitivity and specificity of Focus Diagnostics Rabies Antibody Detection by ELISA when compared to the Bio-Rad Platelia was 82.4 % (CI 72.1–87.9 %), 90.9 % (CI 80.4–96.6 %) and 74.3 % (CI 64.3–79.6 %), respectively. Three false-negative (3/33) and nine false-positive (9/35) samples were found with 66 % (2/3) of the false-negative samples having Bio-Rad Platelia antibody concentrations between 0.50 and 1.00 EU ml\(^{-1}\). A linear regression was performed on the 94 serum samples used in the evaluation (including all equivocal results) (Fig. 3). The analysis produced a regression coefficient of \( R^2 = 0.6328 \) (\( P < 0.0001 \)). A Bland–Altman plot was generated comparing the Focus assay with the Bio-Rad assay (Fig. 4). This analysis produced a plot with an \( R^2 = 0.1603 \) (\( P < 0.0001 \)) and a slope of \(-0.2894\), indicating high variability between the tests.

In general, the DRG assay and the Focus assay were less sensitive than the Bio-Rad Platelia when compared to the RFFIT. It is important to note that the majority of false-negative results were observed in samples with low IU ml\(^{-1}\) values. In this and other studies, samples with moderate or high RFFIT values showed few if any discrepancies with corresponding ELISA antibody concentrations (Briggs et al., 1998). One recent study showed a strong correlation claiming a sensitivity and specificity of 98.96 % and 99.35 %, respectively (Feyssaguet et al., 2007).

The Bio-Rad Platelia showed higher accuracy and specificity than either the DRG or Focus assays. The objective in introducing any ELISA test for rabies compared with a virus-neutralization test is to reduce the number of false-positive results. Therefore, specificity is more important.
than sensitivity. Under these circumstances, false-negative results are not important since the individual can either be revaccinated or have the test repeated using a virus neutralization test. The Bio-Rad Platelia assay showed 100% specificity when non-vaccinated individuals were tested, which is essential to reduce the risk of reporting a protective antibody titre in an unprotected individual. It should be noted that these ELISAs measure all antibody types and do not discriminate the neutralizing antibodies as measured by functional assays (RFFIT and FAVN) and cannot be relied upon to predict the neutralizing activity of the sera.

Although other methods exist to measure rabies-specific antibodies in serum, such as antibody neutralization of lyssaviruses using lentiviral pseudotypes, the results of this study offer further insight into the availability of a faster and more user-friendly method to monitor rabies antibody titres in at-risk individuals following vaccination (Wright et al., 2008). Several ELISA test kits are available and offer a viable alternative to more complex and time-consuming serology methods.

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REFERENCES


