Rabies virus glycoprotein serology ELISA for measurement of neutralizing antibodies in sera of vaccinated human subjects

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ABSTRACT

Background: Vaccination provides protection against infection by inducing VNAs mainly against RABV surface GP. The measurement of VNAs to RABV is commonly used to assess the level of immunity in humans and animals after vaccination. A VNA titer of ≥ 0.5 IU/mL of sera indicates adequate response to vaccination. Here, we report the development and validation of a RABV GP serology ELISA kit for semi-quantitative measurement of VNA titers in sera of vaccinated human subjects.
Methods: Using a recombinant RABV GP expressed in mammalian cells as the capture antigen, the ELISA method was established using HuMAb NM57 reference initially and HRIG reference subsequently. The limit of detection (LOD), linear range, reproducibility, and precision of the method were examined. Specificity and sensitivity were established to assess the diagnostic accuracy.
Results: RABV GP for ELISA plate coating and optimal dilution of human serum sample was 1 μg/mL and 1:20, respectively. Multiple assays were carried out by different technicians at different laboratories for assay standardization. Using the HRIG reference, the LOD was found to be 0.02–0.06 IU/mL and the linear range was 0.2–10.0 IU/ mL. The inter-assay CVs were in the range of 6.60–10.79%, indicating the reproducibility. None of the 12 known negative human sera, tested positive by ELISA, highlighting the specificity. A total of 415 unknown positive human sera were double-blind tested by the RFFIT and ELISA. The VNA titer cut-off value of ELISA was set at 1.5 IU/mL to ensure no false-positive. The diagnostic specificity and sensitivity were 100% and 91.1%, respectively.
Conclusions: The validation data characterize this ELISA as a suitable method for semi-quantitative measurement of VNA titers in human serum samples to assess vaccination status. The ELISA kit can offer simplicity, speed, low cost and high throughput, making it a practical tool for monitoring the immune response following vaccination.

1. Introduction

Rabies continues to pose a public health threat worldwide, especially in developing countries. It is feared for almost 100% fatality rate in humans. The vast majority of human fatalities reported globally came from Africa and Asia, with approximately 40% of cases in children aged <15 years. In most of the world, the major reservoir for human rabies is the dog which is responsible for up to 99% of human rabies cases [1]. Rabies prevention is achieved either by pre- or post-exposure prophylaxis using modern tissue culture rabies vaccines. It happens to be one of the few infectious diseases which can be prevented by vaccination even after exposure [2]. The principal protection against rabies exposure by vaccination is the development of virus neutralizing antibodies (VNAs). The VNAs are primarily directed against the RABV surface GP [3]. Induction of VNAs by the rabies vaccine is a

key determinant of virus neutralization and protection against disease development. This event must occur before the RABV has entered central nerve system [4]. The appropriate administration of modern rabies biologics according to approved regimens virtually assures protection against the development of rabies [5]. The measurement of VNAs to RABV is commonly used to assess the level of immunity to rabies in humans and animals. The WHO and OIE consider rabies vaccination to be adequate when VNA titer is greater than or equal to 0.5 IU/mL in the serum of vaccinated humans and animals [6,7].

Rabies serology methods fall under two categories; *in vivo* method such as mouse neutralization test (MNT) [8] which is obsolete, and *in vitro* methods, such as RFFIT and other cell based functional assays [9,10], cell based binding assays [11] and plate based binding assays/immunoassays [ELISA] [12]. The antibodies targeted by the cell based functional assays are neutralizing whereas by the binding assays are both neutralizing and binding in nature. The cell based functional assays also known as neutralization based assays, though well received and accepted globally, have certain limitations. They require the use of live challenge RABV, containment facility, highly trained technicians and special laboratory infrastructure. In contrast, the ELISA is simpler, affordable and higher in throughput. The ELISA methods using purified virus or vaccine as capture antigens for measurement of VNAs were reported by several researchers worldwide [13–15]. Nevertheless, there was a poor correlation between the antibody titers and protections from RABV challenge [16]. Interestingly, ELISA methods using purified RABV GP offering improved assay specificity have been reported to have better correlation with the RFFIT [17].

Obviously, preparation of enough quantities of native GP is expensive, time consuming and challenging. Many attempts failed to generate mg quantity of purified RABV GP, and have posed hindrance to solve its 3D structure.

Recently, we have developed a Chinese hamster ovarian (CHO) cell line stably expressing RABV GP at 50 mg/L (paper just submitted). Using the mammalian cell expressed RABV GP as the capture antigen in combination with a RABV neutralizing anti-GP HuMAb NM57 [18,19] and WHO-recommended HRIG [20] as standards, we have developed and validated a RABV GP serology ELISA kit for semi-quantitative measurement of the VNA titers against RABV in the sera of vaccinated human subjects. We believe that this RABV GP serology ELISA kit will be a meaningful addition to the existing tool kits meant to address the needs of resource poor countries to combat rabies. The data pertaining to development and validation of RABV GP serology ELISA kit are presented and discussed.

2. Materials and methods

2.1. Reagents and supplies

High-binding 96-well ELISA plates were purchased from Corning, USA. L-glutamine, antibiotics, and TMB substrates were from Sigma-Aldrich, St. Louis, USA. ELISA buffers and solutions were prepared using analytical reagent-grade chemicals unless specified otherwise. Recombinant RABV GP expressed in CHO cell line was obtained from ZhenGe Biotechnology, China. Goat anti-human IgG (H + L) peroxidase conjugate was sourced from Jackson Immunoresearch, USA. FITC conjugated anti-rabies monoclonal globulin was obtained from Fujerebio Diagnostic Inc., USA. Recombinant RABV neutralizing anti-GP HuMAb NM57 with a VNA titer of 883 IU/mg, kindly supplied by Huabei Pharmaceuticals, Shijiazhuang, China and the HRIG sourced from the EDQM were used as the reference standards.

2.2. Rabies virus GP serology ELISA kit

The RABV GP serology ELISA is based on the principle reported elsewhere, albeit with necessary modifications [13]. Briefly, known amount of recombinant RABV GP was diluted in PBS (10 mM, pH 7.4) and 100 μL of the solution was added to each well of 96-well high binding ELISA plates (8 wells/strip, Corning, USA) and incubated overnight at 2–8 °C. The wells were emptied and washed twice with PBS and unsaturated sites were blocked with 3% BSA in PBS by incubating for 1 h at room temperature. The coated plates were air-dried and sealed in plastic bags and stored at 2–8 °C until used.

Anti-RABV GP neutralizing mAb HuMAb or HRIG standards were first diluted in negative human serum (pooled serum from 12 human subjects who never been vaccinated against rabies). Prior to test, human serum samples and the standards were diluted in sample dilution buffer, 20% Calf-serum (CS) in PBS. 100 μL of appropriately diluted sample was added to each well of the RABV GP-coated plates and incubated for 1 h at 37 °C with constant shaking. The wells were emptied and washed twice with PBS. 100 μL of appropriately diluted goat anti-human IgG (H + L) peroxidase conjugate in 3% BSA-PBS was added to the respective wells and incubated for another 1 h at 37 °C with constant shaking. The wells were emptied and washed five times with PBS containing 0.1% Tween 20 (PBS-T) before addition of TMB substrate solution. The chromogenic development was stopped using 0.1 M H_2SO_4 after 15 min of incubation in the dark. Optical density (OD) was measured at 450 nm wave length in a microplate spectrophotometer (Thermo Scientific, Multiskan MK3). The quantity of anti-RABV GP antibody was calculated using the standard curve of the known amount of HuMAb NM57 or HRIG.

2.3. Rapid fluorescent focus inhibition test (RFFIT)

BSR cells were cultured in Dulbecco's Modified Eagle's Medium/ Nutrient Ham F12 (DMEM F12) with 10% heat-inactivated FBS and antibiotics at 37 °C in a 5% CO_2 atmosphere. Challenge virus standard (CVS 11) RABV strain was grown in BSR cells. The dose of CVS 11 RABV strain which caused 80% infection of BSR cells after 24 h was mixed with serial dilutions of the sera to be titrated. A reference serum adjusted to 30 IU/mL was included in each test. After 1 h of incubation at 37 °C, 200 μL of the freshly trypsinized BSR cells was added to each well. After 24 h of incubation at 37 °C in a 5% CO_2 atmosphere, the cells were washed and fixed, then incubated with FITC-conjugated anti-rabies monoclonal antibody from Fujerebio. The percentage of infected cells at each serum dilution was determined. This allowed the determination of the VNA titer of the test human sera by comparison with the reference serum. The VNA titer of the test human sera was expressed in terms of IU/mL as practiced universally.

2.4. Serum samples for assay

Negative serum samples were obtained from human subjects (n = 12) that never received rabies vaccination but vaccinated with HBV, tetanus, and many other vaccines previously. Serum samples were also obtained from human subjects (n = 415) that received rabies vaccination. Informed consent was obtained from all the human subjects who participated in the study after the nature and possible consequences of this study had been fully explained and the protocols were approved by the institutional ethical committee. The serum samples were split into two parts after inactivation at 56 °C for 30 min, one being used for the ELISA assay for specificity, sensitivity and correlation, another for the RFFIT assay, using HRIG as the reference. The serum samples were stored at −20 °C until used. For ELISA, each serum sample, including the

QC sera (0, 0.5 and 1.5 IU/mL of HRIG) was tested in duplicates and 45 samples can be accommodated on one plate. The VNA levels were semi-quantitatively determined by OD interpolation against the two positive QC sera. Samples with OD_{450} values less than the one from 0.5 IU/mL HRIG QC serum were assigned a VNA titer of <0.5 IU/mL; greater than or equal to the one from 1.5 IU/mL of HRIG QC serum were assigned a VNA titer of $\geq$1.5 IU/mL; greater than or equal to the one from 0.5 IU/mL of HRIG QC serum but less than the one from 1.5 IU/mL of HRIG QC serum were assigned a VNA titer of 0.5–1.49 IU/mL. The assay acceptance criteria (AAC) are OD_{450} value for 0.5 IU/mL (ranging from 0.64 to 0.80) and 1.5 IU/mL (ranging from 1.35 to 1.80).

2.5. Data analysis

The mean QC value for each test was calculated according to standard curve of the HRIG. Normalized data were used for statistical analysis with Sigmaplot [21]. ROC curves were used to evaluate correlation between ELISA and RFFIT. The confidence interval was calculated by assuming a binomial distribution. The test data were expressed in the form of a Levey-Jennings graph.

3. Results

3.1. Determination of optimal concentration of RABV GP for coating

Fatal nature of the rabies necessitates the generation of a VNA response sufficient enough to confer protection. Standardization of assays including both assay components and test conditions are highly recommended [20].

To evaluate the effect of RABV GP coating concentration for capturing anti-RABV antibodies in test serum sample, each well of 96-well ELISA plate was coated with 100 μL of RABV GP at three different concentrations (0.1, 0.3 and 1 μg/mL) in 10 mM PBS pH 7.4 at 2–8 °C overnight. The solid phase RABV GP was probed using appropriately diluted humanized RABV neutralizing anti-GP monoclonal antibody HuMAb NM57 (0, 25, 100, 400, 1600, 6400, 25600, 102400 ng/mL) in negative human serum (pooled serum from 12 human subjects who never been immunized with rabies vaccine). The resulting immune complex was detected using peroxidase conjugated goat anti-human IgG (H + L) antibodies to target both IgG and IgM antibodies. As shown in Table 1, at all three different coating concentrations of RABV GP, the OD values showed dose dependency, while the maximal OD values increased with increasing coating concentration of RABV GP. Since the background OD values did not change significantly, 1 μg/mL concentration of RABV GP was considered optimal for coating of ELISA plate for kit manufacture.

3.2. Determination of optimal dilution of human serum samples

There is a need to dilute test human serum samples since matrix components, especially the host antibodies, can contribute to high assay background if undiluted. A set of anti-RABV GP HuMAb NM57 standards (3200, 1600, 800, 400, 200, 100, 50 ng/mL) were tested at two different dilutions to experimentally determine the optimal dilution of human serum samples. As shown in Table 2, using 20% calf serum (CS)-PBS as diluent, the overall signal-to-noise ratio was found to be better with 1:20 dilution.

3.3. Determination of the LOD and linearity of the kit

Limit of Detection (LOD): The LOD is defined as being the mean absorbance value of a blank sample (pooled negative human sera) in duplicate plus three times the standard deviation [22].

Table 1
Concentration of RABV GP for plate coating.

| NM57 (ng/mL) | Coating Conc. | | | | | |
	0.1 μg/mL (OD_{450})		0.3 μg/mL (OD_{450})		1 μg/mL (OD_{450})	
102,400	2.046	1.993	2.722	2.509	2.494	2.795
25,600	1.542	1.539	2.665	2.491	2.569	2.777
6400	1.039	1.032	1.902	1.887	2.689	2.689
1600	0.75	0.731	1.121	1.041	1.946	2.011
400	0.654	0.621	0.709	0.73	0.958	0.959
100	0.568	0.601	0.601	0.599	0.625	0.606
25	0.597	0.528	0.589	0.611	0.56	0.497
0	0.573	0.501	0.616	0.602	0.506	0.5

Note: Each well was coated with 100 μL of RABV GP at three different concentrations, probed with different concentrations of RABV neutralizing anti-GP MAb HuMAb NM57 standards prepared in 1% negative human serum-PBS. After washes, the immune complexes were detected with Goat anti-human IgG (H + L) peroxidase conjugate (1:5000 in 3% BSA-PBS). TMB substrate solution was added and OD measured at 450 nm wave length in a microplate spectrophotometer.

Table 2
Determination of optimal dilution of human serum samples.

| NM57 (ng/mL) | Sample dilution | | | |
	1:20 (OD_{450})		1:40 (OD_{450})	
3200	1.888	1.86	1.537	1.496
1600	1.501	1.463	0.979	1.063
800	1.017	1.013	0.681	0.652
400	0.713	0.706	0.44	0.424
200	0.51	0.531	0.34	0.333
100	0.421	0.424	0.283	0.267
50	0.375	0.398	0.251	0.259
0	0.333	0.351	0.273	0.242

Note: Each well was coated with 1 μg/mL RABV GP, 100 μL of 1:20 or 1:40 diluted reference HuMAb NM57 standards (0–3200 ng/mL spiked in pooled negative human sera) in 20% CS-PBS were added to each well. After the incubation, wells were washed and probed with goat anti-Human IgG (H + L) peroxidase conjugate (1:5000 in 3% BSA-PBS). After wash, TMB substrate solution was added for colour development and OD measured at 450 nm wave length in a microplate spectrophotometer.
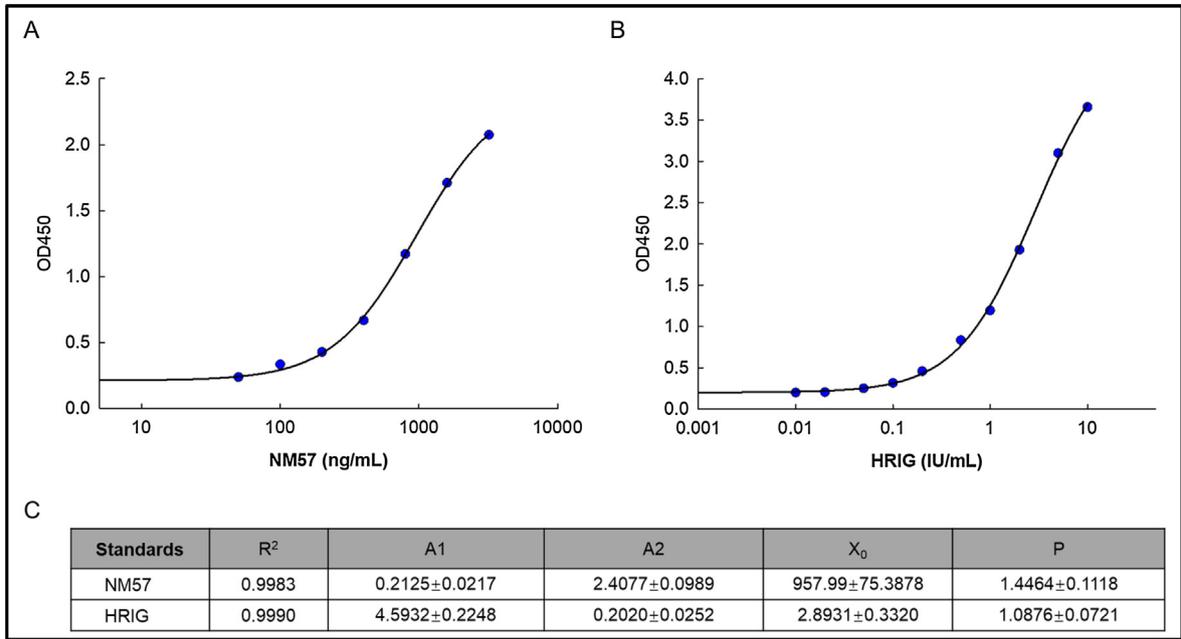
Fig. 1. 4-PL curve using either NM57 or HRIG as standards. 4-PL curves of HuMAb NM57 and HRIG standards. 100 μL of 1:20 diluted reference HuMAb NM57 or HRIG standards at different concentrations were added to each well coated with RABV GP. Two 4-PL curves (A & B) was plotted by having concentrations of NM57 or HRIG standards on 'X axis' and OD values on 'Y axis'. [Equation: $y = A2 + (A1-A2)/(1 + (X/X_0)^P)$] C. The characteristics of two 4-PL curves are listed, A1 indicated the estimation of asymptotes under curves, A2 referred to the estimation of asymptotes on curves, X_0 refers to the concentration for 50% of maximal effect, and P represented the slope of curve.
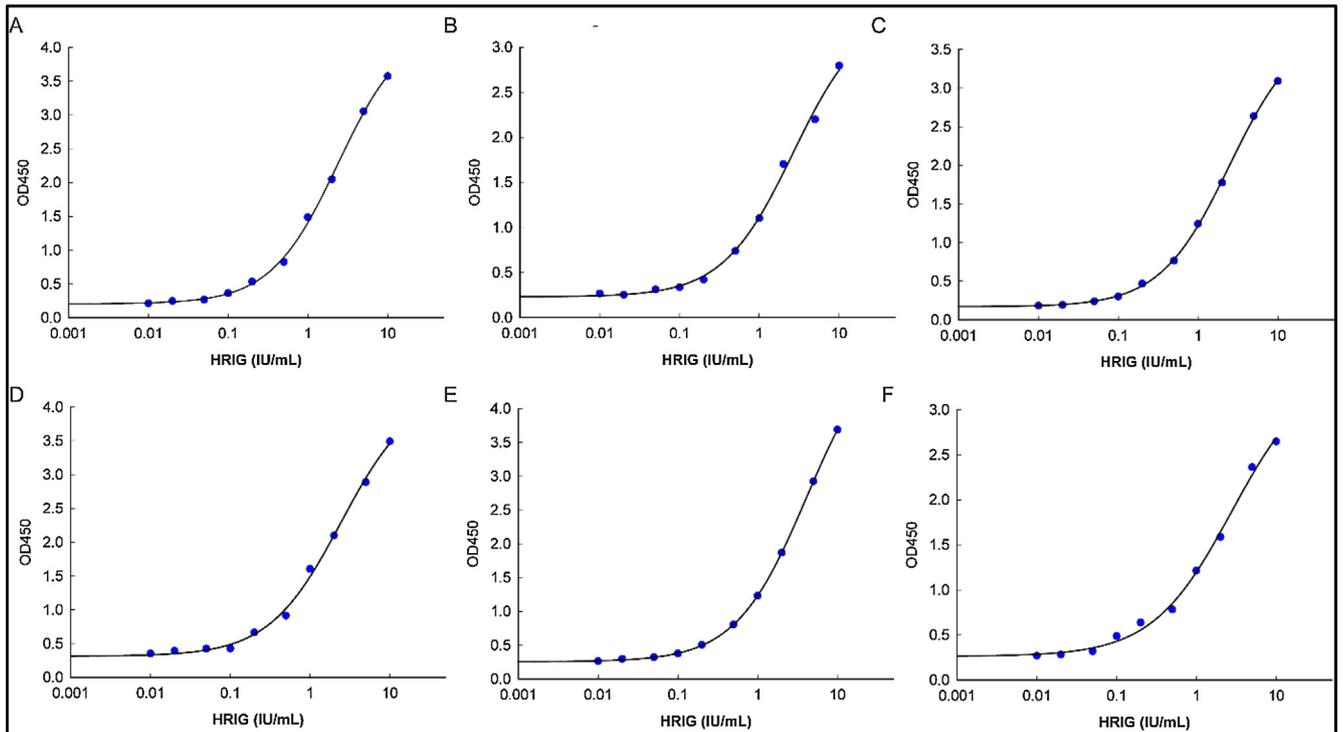
Fig. 2. 4-PL curves of six different test runs using HRIG as the standards. 4-PL curves of the HRIG standards. 100 μL of 1:20 diluted HRIG standards (0–10.00 IU/mL spiked in negative human serum) in 20% CS-PBS were added to each well coated with RABV GP. Six curves (A-F) came from six runs altogether performed by three people on two different days. The 4-PL curves were plotted by having concentrations of the HRIG standards on 'X axis' and OD values on 'Y axis' [Equation: $y = A2 + (A1-A2)/(1 + (X/X_0)^P)$].

Linearity: It was determined by using the linear correlation coefficient of the standard curve established with the references either RABV neutralizing HuMAb NM57 standard samples (0, 50, 100, 200, 400, 800, 1600, 3200 ng/mL) or HRIG (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 IU/mL). Each reference standard was tested in dupli-

cate by ELISA. The linearity was calculated by plotting a 4-PL standard curve by having concentration of reference standards on 'X axis' and OD values on 'Y axis'. As shown in Fig. 1A, using HuMAb NM57 as standards, the LOD of RABV GP ELISA was 44 ng/mL with a linear range between 50 and 3200 ng/mL, and $R^2 = 0.9983$. Also

Table 3
LOD and linear range of assay using HRIG as standards.

HRIG	Test run 1	Test run 2	Tes runt 3	Test run 4	Test run 5	Test run 6
LOD (IU/mL)	0.0491	0.0529	0.0196	0.0516	0.0319	0.0339
Linear range (IU/mL)	0.2–10	0.2–10	0.2–10	0.2–10	0.2–10	0.2–10
R^2	0.9999	0.9969	0.9997	0.9966	0.9990	0.9990

Note: Six test runs were carried out by three different technicians on two different days. 100 μL of 1:20 diluted reference HRIG standards (0–10 IU/mL spiked in negative human serum) in 20% CS-PBS were added to each well coated with RABV GP. LOD, Limit of Detection, is derived from the formula: LOD = NC + 3SD (NC indicated average OD readings of duplicate negative control wells and SD represented the standard deviation). R^2 is adjusted R-square, represents the correlation co-efficient of each curve.
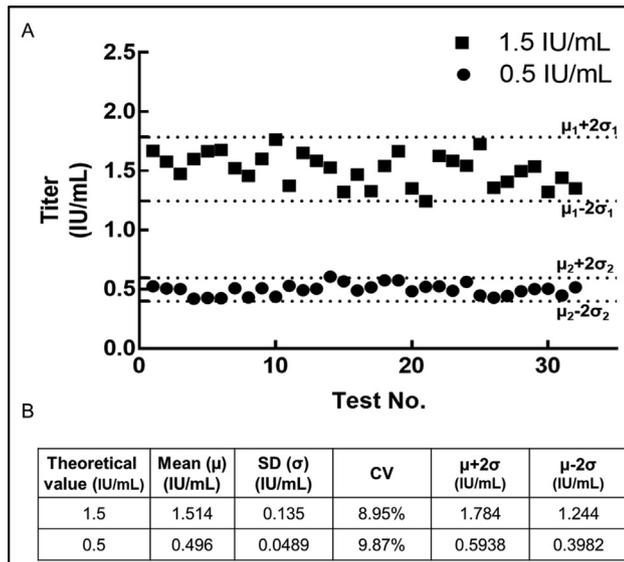
Fig. 3. Levey-Jennings graphs for quality control. Levey-Jennings graphs for monitoring performance of the ELISA using the HRIG. A. Two HRIG QC sera at 0.5 IU/mL and 1.5 IU/mL were tested for 30 times and the VNA titers calculated from the standard curves. μ denotes the mean of calculated VNA titers and σ represents the standard deviation. Each data point represents the mean of each QC serum tested in duplicate. B. Statistics obtained from 30 test runs including the mean and standard deviation.

shown in Fig. 1B, using HRIG as standards, the ELISA's LOD was 0.0529 IU/mL with a linear range between 0.2 and 10 IU/mL and $R^2 = 0.9969$.

Based on above data, the best manufacturing and key testing parameters for the RABV GP serology ELISA kit were selected as (1) 1 μg/mL RABV GP for plate coating, (2) 1:20 dilution of human sera using 20% CS-PBS as sample diluent, (3) 1:5000 dilution of goat anti-human IgG (H + L) peroxidase conjugate using 3% BSA/PBS as diluent for detection.

3.4. Reproducibility: Intra-assay and inter-assay precisions

Several batches of the RABV GP serology ELISA kits were manufactured at two different locations, and used by different technicians on different days for assessing the reproducibility of

manufacturing and testing. The reference HRIG standards (0–10 IU/mL) were tested independently by two technicians on three different days employing kits belonging to two different batches. As shown in Fig. 2 and Table 3, the LODs of six test runs were all less than 0.1 IU/mL and the linear ranges were from 0.2 to 10 IU/mL with $R^2 \geq 0.9966$. Also shown in Fig. 3, two QC sera of HRIG at 0.5 and 1.5 IU/mL were tested for 30 times on the same plates and the VNA titers were calculated from the standard curves. The intra-assay precision of samples (%CV) were in the range of 8.95–9.87%.

To assess the inter-assay precision of samples (CV), three QC sera of HRIG at 0.5, 1.0 and 2.0 IU/mL were examined in duplicate by three different technicians and the VNA titers were calculated from the standard curves. Summarized in Table 4, all three CVs were in the range of 6.60–10.79%, which is within the acceptable criterion of less than or equal to 15%.

3.5. Correlation between RABV GP serology ELISA and RFFIT

The diagnostic specificity of the kit was demonstrated by testing 12 serum samples derived from humans that received vaccines other than rabies vaccine (HBV, TT, DT, IPV, etc). The 12 serum samples together with one positive control were double blind tested by RFFIT and ELISA at two independent laboratories situated in different locations. While the positive control turned positive, all 12 test serum samples tested negative i.e., VNA titer less than 0.5 IU/mL by both RFFIT and ELISA, showing 100% specificity (see Table 5).

In addition, a total of 415 samples from rabies vaccinated human subjects were double-blind tested using both the methods (RFFIT and ELISA). For ELISA, in addition to test samples, 3 HRIG QC sera (0, 0.50, 1.50 IU/mL) were included on each 96-well plate. As shown in Table 6.1, 38 samples were determined with VNA titers less than 0.5 IU/mL, 58 samples were with VNA titers between 0.50 and 1.49 IU/mL and 319 samples were ≥1.50 IU/mL by ELISA. Fifteen out of 38 samples with ELISA VNA titers <0.5 IU/mL were in agreement with RFFIT assay. On the other hand, 4 samples with RFFIT VNA titers between 0.20 and 0.49 IU/mL were determined to be between 0.50 and 1.49 IU/mL by ELISA.

As shown in Table 6.2, if we set the cut-off value at 0.5 IU/mL, 4 of the 19 RFFIT-negative samples would be falsely reported as positive by ELISA, with specificity at 78.9%. Since rabies is a fatal disease and a false positive indication of a sufficient VNA response could be a disaster to the patient and it is not acceptable. Therefore,

Table 4
Inter-assay CV.

Theoretical Titer (IU/mL)	Calculated Titer (IU/mL)						CV (%)
	Test run 1	Test run 2	Test run 3	Test run 4	Test run 5	Test run 6	
2.0	2.27	1.94	1.99	1.94	2.00	1.91	6.60
1.0	0.98	1.03	1.15	0.93	0.99	1.09	7.78
0.5	0.49	0.48	0.41	0.56	0.5	0.44	10.79

Note: Six test runs were carried out by three different technicians on two different days. Three HRIG standards at 0.5, 1.0 and 2.0 IU/mL were tested and the titers were calculated from the standard curves.

Table 5
Specificity of ELISA.

		RFFIT		
		Positive	Negative	Sum
ELISA	Positive	1	0	1
	Negative	0	12	12
	Sum	1	12	13

Note: 12 human serum samples from people never vaccinated against rabies and one HRIG positive control (0.5 IU/mL) were assayed by RFFIT and ELISA. For ELISA, the positive indicated that the OD_{450} of serum samples is greater than or equal to the OD_{450} of HRIG standard (0.5 IU/mL).

Table 6.1
VNA titers of rabies vaccinated patients.

		ELISA (IU/mL)			
		<0.50	0.50–1.49	≥1.50	Total
RFFIT (IU/mL)	<0.20	5	0	0	5
	0.20–0.49	10	4	0	14
	0.50–0.99	9	20	0	29
	1.00–1.49	5	12	0	17
	≥1.50	9	22	319	350
	Total	38	58	319	415

Table 6.2
Sensitivity and specificity of ELISA in comparison with RFFIT.

Cut-off (IU/mL)	Sensitivity	Specificity	Total coincidence rate
0.50	94.2% (373/396)	78.9% (15/19)	93.5% (388/415)
1.50	91.1% (319/350)	100% (65/65)	92.5% (384/415)

Note: 415 human serum samples from people vaccinated against rabies were double-blind tested by RFFIT and ELISA. For each 96-well plate assay, in addition to test samples, 3 HRIG QC controls (0, 0.50, 1.50 IU/mL) were included. For sample with OD_{450} less than the one from 0.50 IU/mL of HRIG QC, its VNA titer is defined as less than 0.50 IU/mL. For sample with OD_{450} greater than or equal to the one from 0.50 IU/mL of HRIG QC, but less than the one from 1.50 IU/mL of HRIG QC, its VNA titer is defined as 0.50–1.49 IU/mL. For sample with OD_{450} greater than or equal to the one from 1.50 IU/mL of HRIG QC, its VNA titer is defined as ≥1.50 IU/mL.

we prefer to set the cut-off value at 1.5 IU/mL, although the sensitivity dropped from 94.2% to 91.1%, the specificity reached 100%, with a total coincidence rate of 92.5%.

The VNA titres obtained by ELISA were compared with the ones from RFFIT at two different cut-off values. At cut-off of 0.5 IU/mL, the ROC curve area was 0.9499, with a 95% confidence interval from 0.9218 to 0.9780, and the P value is under 0.0001. At cut-off of 1.5 IU/mL, the ROC curve area was 0.9723, with a 95% confidence interval from 0.9580 to 0.9866, and the P value is under 0.0001 (see Fig. 4).

Based on the above data, with the cut-off value set at 1.5 IU/mL, the ELISA kit meets the criterion for intended application of first-line quick screening. For those patients with ELISA VNA titers between 0.5 and 1.49 IU/mL, we would recommend them to receive an additional vaccine boost.

4. Discussion

Vaccine preventable nature of deadly diseases like rabies, makes disease prevention quite straight forward. Quality controlled modern cell culture rabies vaccines are made available and accessible to the global population at risk. The presence of 0.5 IU/mL of VNAs in serum is considered as a reliable indicator of adequate immune response to vaccination [23–26] ensuring satisfactory sero-conversion against rabies [26,27]. Cell based functional assays such as RFFIT and FAVN are the two classical reference assays prescribed by the WHO and OIE. However, alternative assays have been developed essentially to overcome the limitations associated with the classical assays. Reliability, low cost, high throughput and short turnaround time are the key drivers of development of alternative rabies serology assays. Irrespective, the serology assays are primarily used to determine the immunization status of subjects that have received rabies vaccination. Additionally, they are useful for evaluation of vaccines or new schedules of rabies prophylaxis, determination of immunization status of animals prior to importation to rabies-free countries, screening of plasma donors for RIG production and benchmarking of newly developed serological methods [13].
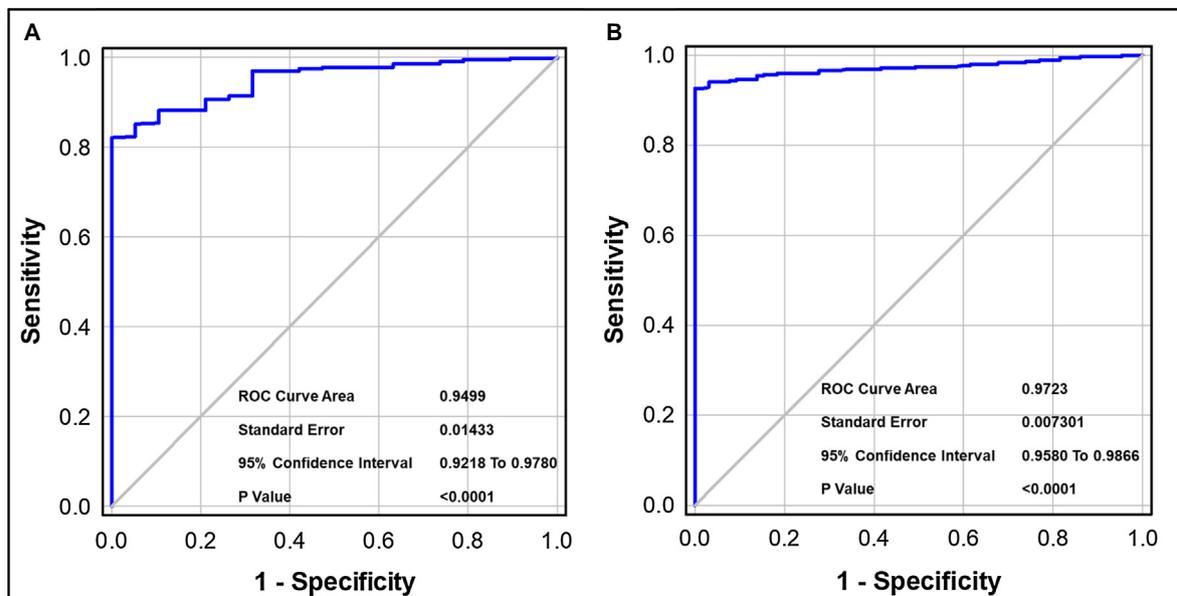
Fig. 4. ROC curve analysis of ELISA and RFFIT. The VNA titres obtained by ELISA was fit with results of RFFIT at two different cut-off values. A. The statistics obtained from the ROC curve analysis at cut-off of 0.5 IU/mL. The curves were plotted by having 1-specificity on 'X axis' and sensitivity on 'Y axis'. The sensitivity denoted the positive coincidence rate and specificity denoted the negative coincidence rate. B. The statistics obtained from the ROC curve analysis at cut-off of 1.5 IU/mL.

Indirect ELISA is one of the simple assay formats available for measurement of antibodies specific to antigenic targets in biological samples e.g. serum. Serum antibodies are trapped using a relevant antigen immobilized on the solid phase. The solid phase antigens such as purified whole virus (inactivated) antigen, freeze-dried vaccine antigen and purified RABV subunit e.g. GP and RABV GP expressing transformed cell line have been evaluated for serology assays thus far. However, purified RABV GP is better than the whole virus antigens in terms of highly defined nature, homogeneity and lack of interfering substances. It also provides a better evaluation of specific neutralizing antibodies [16]. In that, natively folded RABV GP biochemically purified from inactivated whole virus antigen has been in use for quite sometime. However, recombinant RABV GP expressed in eukaryotic cells can potentially replace the conventional RABV GP when structural and functional characteristics are found to be comparable. In this study, we have shown that there exists a typical dose dependency between RABV GP concentration and the binding of a humanized RABV neutralizing anti-GP HuMAb NM57. It is evident from the results that 1 μg/mL concentration of recombinant RABV GP was found to be ideal for coating the ELISA plates. At an expression level of 50 mg/L, the cost of recombinant RABV GP coated per 96-well plate worked out cheaper than the cost of the plate itself.

It was determined that the optimal dilution of human sera is 1:20 using 20% CS-PBS as a diluent. Such high dilution of sera, in addition to reduction of matrix complication, reduced the volume of serum samples from 2 to 3 mL blood for RFFIT, to be drawn by nurses or professionals at clinic or hospital, to 4–5 drops of blood which can be done at home by the patients themselves. It adds extra convenience.

The RABV GP serology ELISA kit was standardized for manufacturing and assay parameters such as linearity, LOD, precision (inter- and intra-assay), and reliability of production were evaluated. The validated RABV GP serology ELISA kit is found to be fit for purpose i.e. semi-quantitative measurement of VNA concentration in serum samples derived from vaccinated human subjects.

This RABV GP serology ELISA kit was the product of collaborations of national laboratories, regulatory agencies, as well as commercial manufacturer. The RABV GP serology ELISA kits have been manufactured at a GMP facility with a capacity of 100,000 kits per year, enough to perform 4,500,000 tests. Leading by the Chinese CDC, in collaborations with more than 10 hospitals, a post-market study of at least three different rabies vaccines enrolling tens of thousands of patients will be carried out to understand the relationship between *in vitro* measurement and *in vivo* protection.

5. Conclusions

The validation data characterize this ELISA as a suitable method for semi-quantitative measurement of VNA titer in human serum samples as a part of assessment of their vaccination status. The RABV GP serology ELISA kit can offer simplicity, speed, low cost and high throughput, making it a practical tool for monitoring the immune response of human subjects following mass rabies vaccination campaigns.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.08.043.

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