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G-protein based ELISA as a potency test for rabies vaccines

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ABSTRACT

The NIH test is currently used to assess the potency of rabies vaccine, a key criterion for vaccine release. This test is based on mice immunization followed by intracerebral viral challenge. As part of global efforts to reduce animal experimentation and in the framework of the development of Sanofi Pasteur next generation, highly-purified vaccine, produced without any material of human or animal origin, we developed an ELISA as an alternative to the NIH test. This ELISA is based on monoclonal antibodies recognizing specifically the native form of the viral G-protein, the major antigen that induces neutralizing antibody response to rabies virus. We show here that our ELISA is able to distinguish between potent and different types of sub-potent vaccine lots. Satisfactory agreement was observed between the ELISA and the NIH test in the determination of the vaccine titer and their capacity to discern conform from non-conform batches. Our ELISA meets the criteria for a stability-indicating assay and has been successfully used to develop the new generation of rabies vaccine candidates. After an EPAA international pre-collaborative study, this ELISA was selected as the assay of choice for the EDQM collaborative study aimed at replacing the rabies vaccine NIH *in vivo* potency test.

1. Introduction

Rabies is a viral disease of the central nervous system caused by rabies viruses that is invariably fatal after clinical signs appear [1]. Rabies disease can be prevented using human vaccines in a pre- and post-exposure context. The rabies vaccine production process includes a potency testing step to ensure that these vaccines have the capacity to induce a protective immune response in vaccinated individuals [2]. Potency testing of inactivated rabies vaccines is traditionally performed using intra-cerebral challenge in mice. The method was developed by the National Institute of Health (NIH) and is currently required for vaccine release [2,3].

The NIH test has a number of limitations such as the use of mice and virulent virus, its long duration and cumbersome nature [4]. During the International Workshop on Alternative Methods for Human and Veterinary Rabies Vaccine Testing in 2011 (Ames, USA), worldwide rabies experts agreed on the need to replace the NIH test by an enzyme-linked immunosorbent assay (ELISA) [5]. The European monograph (Pharmacopoeia, 07/2014) additionally recommends an immunochemical method, such as a Single Radial Immunodiffusion (SRID) assay, or tests based on antibody binding to assess vaccine potency as well as antigen content.

Several ELISAs have been proposed as alternatives to the NIH test, such as a method for rabies G-protein estimation using monoclonal antibodies (mAbs) directed against site III [6,7] or site II [8,9] of this protein. Both sites were identified as important to achieve protection against the virus [10,11]. Assuming that the mAb recognizes a correctly folded G-protein, the G-protein content is indicative of the vaccine potency.

Two rabies vaccines manufactured by Sanofi Pasteur are licensed, the Human Diploid Cell Rabies Vaccine (HDCV or, Imovax rabies™) and the Purified Vero Cell Rabies Vaccine (PVRV or Verorab®). Sanofi Pasteur has improved the current Verorab® vaccine to develop a next generation, highly-purified vaccine, produced without any material of human or animal origin (next-generation purified Vero cell rabies vaccine [PVRV-NG]). During this vaccine optimization production process, we developed and validated an ELISA which can be used as release test and which is described here.

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This ELISA is based on two neutralizing mAbs that target G-proteins sites II and III and has been used to monitor vaccine blending. The ELISA detects specifically the native form of the G-protein and does not respond to unfolded or chemically modified forms, thus discriminating between potent and sub-potent vaccine lots.

2. Materials and methods

2.1. Materials

2.1.1. Monoclonal antibodies

MAb D1-25 was obtained from P. Perrin (Pasteur Institute, France) [12,13] and mAb WI 1112 from the Wistar Institute (also referred to in the literature as 1112 or TJU 1112 from B. Dietzschold, Jefferson University, USA) [14,15]. Biotinylated mAb D1-25 was obtained by coupling biotin *N*-hydroxysuccinimide ester to primary amine groups [16]. Biotinylated mAb was diluted in 50% (v/v) glycerol and stored at −20 °C. Surface Plasmon Resonance (SPR) experiment was performed to verify that biotinylation did not affect virus recognition (Supp. Fig. 1.).

2.1.2. Reference antigen

In-house produced bulk vaccine lots were used as an internal reference, which was titrated against the WHO International Rabies standard (NIBSC ref. 07/162).

2.1.3. Rabies vaccine samples

All vaccine batches used in the study correspond to PVRV-NG batches produced in-house.

2.1.4. Virus strains

Flury LEP, Challenge Virus Standard (CVS11) and Pitman-Moore (PM) lyssavirus strains are viral suspensions from Sanofi Pasteur produced from different cell lines (VERO for Flury LEP, BHK-21 for CVS and MRC-5 for PM).

2.2. Methods

2.2.1. Determination of monoclonal antibody neutralizing activity

Neutralizing rabies-specific mAb titers were determined by the Rapid Focus Fluorescent Inhibition Test (RFFIT) using the Nunc™ Lab-Tek™ II chamber slide™ system (ThermoFischer scientific, ref 154534). Approximately 100 $CCID_{50}$ of challenge rabies virus (Flury LEP, CVS11 and PM) in 100 µl were added to 100 µl of successive dilutions of mAbs and incubated for 90 min in a humidified cell culture incubator (36 °C; 5% CO_2). BHK-21 cells (100 µl; 10^5 cells/ml [9]), were then added to the virus/mAb mixture and incubated for 24 h. Non-neutralized virus was detected by staining BHK-21 cells with FITC anti-rabies monoclonal globulin (Fujirebio Diagnostics Inc., ref. 800-092). Titers were determined by comparing the results obtained for the mAbs with those obtained using the WHO international standard for anti-Rabies Immunoglobulin (NIBSC code: RAI). Neutralizing potency was determined by comparing the 50% neutralizing titer for the mAb to the 50% neutralizing titer of the standard (2 IU/ml).

2.2.2. Generation of modified vaccine batches

2.2.2.1. Reduction/alkylation of inactivated virus. Inactivated virus (vaccine bulk; 300 µg/ml) was dialyzed against buffer A (20 mM Tris, 150 mM NaCl, pH 8.0) using 10 kDa cut-off membranes (Slide-A-Lyzer™ Dialysis Cassettes, ThermoFischer Scientific); dithiothreitol was added to a final concentration of 30 mM. The resulting samples were incubated at 37 °C under stirring for 60 min, then at 4 °C for 5 min. Iodoacetamide was then added to a final concentration of 120 mM. The mixture was kept at room temperature, under stirring and in darkness, for 30 min, then dialyzed exhaustively against buffer A and stored at 4 °C.

2.2.2.2. Excessive inactivation using beta-propiolactone. Bulk samples of non-inactivated virus were inactivated using beta-propiolactone (BPL) at various final concentrations superior to those used in vaccine production, i.e. 1/2000 to 1/500 (v/v) vs 1/4000 (v/v). Samples were incubated at 12 °C for 32 h with the mixtures containing different BPL concentrations. BPL was neutralized by the addition of sodium thiosulfate (80 g/L) and the mixture was incubated at 37 °C for 2.5 h. Samples were then concentrated to 150 µg protein/ml using Amicon® Ultra Centrifugal Filters with a 10 kDa cut-off (Millipore).

2.2.2.3. Heat treatment of inactivated virus. Vaccine doses (lyophilized product) were incubated at 65 °C for 3 days. Product was reconstituted in 0.4% (w/v) sodium chloride solution. A mixture of equal volume of heated and non-heated product was made to generate sub-potent batches.

2.2.2.4. ELISA. Maxisorp ELISA plates (Nunc) were coated with mAb WI 1112 in carbonate–bicarbonate buffer (pH 9.6, 100 µl/well) and incubated at 4 °C for 16 h. Plates were then washed three times with PBS containing 0.05% (v/v) Tween-20 and blocked with 100 µl/well of 1% (w/v) bovine serum albumin (BSA) in PBS at 37 °C for 1 h. Standard antigen or rabies vaccine dilutions (100 µl) were added and plates were incubated at 37 °C for 1 h. Plates were then washed and 100 µl of dilution buffer (PBS, 0.05% (v/v) Tween-20, 0.1% (w/v) BSA) containing biotin-labelled mAb D1-25 were added to each well, followed by incubation at 37 °C for 1 h. Dilution buffer (100 µl) containing streptavidin-peroxidase polymer (Southern Biotechnology Associates) was added to each well and incubated at 37 °C for 1 h. After washing, 100 µl of chromogen substrate solution (4 mg/mL O-phenylenediamine, Sigma–Aldrich) in 0.05 M citrate buffer, pH 5.0, 0.009% (v/v) H_2O_2 was added; plates were incubated in the dark at room temperature for 30 min. The reaction was stopped by adding 50 µl of 2N sulphuric acid. The OD_{492nm} was determined using an ELISA reader (Molecular Devices). The G-protein content, estimated as international units (IU/ml), was calculated by the parallel line method, according to the European Pharmacopoeia [17].

2.2.2.5. ELISA validation. Specificity was assessed using the vaccine final bulk matrix (i.e. not containing antigen) and spiking the antigen of interest. Standard rabies vaccine (WHO International standard, NIBSC code: 07/162) was spiked in matrix. Linearity and accuracy assessment was carried out by triplicate analyses on 5 test formulations containing different quantities of standard rabies vaccine. Repeatability was assessed by conducting sextuple replicates by the same operator on the same day. These 6 independent titrations were repeated 3 times by different operators, on different days to assess intermediate precision.

2.2.2.6. Mouse potency assay. Mouse potency tests were performed according to the European Pharmacopoeia guidelines [3]. The assay was conducted in accordance with the EU Directive 2010/63/EU for animal experiments. The NIH test involved intraperitoneal injection (500 µl) of mice (16 OF-1 mice weighting from 11 to 15 g per dilution) with vaccine dilutions 1/25, 1/125, 1/625 and 1/3125. After immunizations at days 0 and 7, mice were challenged at day 14 with an intracerebral injection (30 µl) of 30-fold the lethal dose of rabies strain CVS11. Mice were observed until day 28 and the number of animals surviving were used to calculate the ED_{50} of the vaccine, which is normalized with the ED_{50} of the reference (internal reference calibrated against WHO International standard NIBSC ref

07/162) to obtain a titer in IU NIH/dose. To note that the human endpoint as mentioned and accepted by European Pharmacopoeia is used for animal observation and mice survival assessment.

3. Results

3.1. mAbs D1-25 and WI 1112 neutralize several rabies virus strains

The ELISA described here is based on the capacity of mAbs to bind specifically to the native G-protein. MAb D1-25 recognizes the antigenic site III which is one of the major immune-dominant epitopes [18]. MAb WI 1112 recognizes the antigenic site II formed by a conformational and discontinuous epitope linked by disulfide bridging [19,20]. These mAbs neutralized the 3 rabies strains tested, namely CVS11, Pitman More and Flury LEP (Table 1). MAb WI 1112 had a significantly higher neutralizing activity than mAb D1-25. In addition, the neutralizing capacity of a given mAb was comparable for the three virus strains.

3.2. Virus modification dramatically reduces the ELISA titer

We generated modified virus samples by either protein reduction/alkylation or BPL inactivation using doses that were significantly higher than those used for vaccine production. SDS-PAGE analysis of these modified samples revealed no significant changes in the protein content (Supp. Fig. 2). Virus reduction treatment modifies G protein conformation as demonstrated by Differential Scanning Calorimetry (DSC) analysis as previously published [21]. Regular BPL treatment (1/4000) does not seem to alter G protein conformation [21]. Evaluation by electron microscopy revealed significant alterations in viral structure for both type of samples reduced/alkylated and BPL overinactivated (Supp. Fig. 3).

Both types of modified virus showed a significant decrease in the ELISA titer (Table 2). There was an agreement between the ELISA response obtained with BPL-inactivated samples and the BPL dose used for inactivation. This indicates that the mAbs used in our ELISA are able to discriminate native from altered structure of the G protein.

3.3. Validation of the ELISA

Our ELISA was validated according to the guidelines of the International Conference on Harmonisation of Technical Requirements for registration of Pharmaceuticals for Human Use [22]. Specificity, linearity, accuracy and precision were assessed. The data obtained for linearity were also used to evaluate the accuracy of the assay and to determine the measurement interval.

Validation using only the matrix (i.e. no antigen) showed high specificity of the assay, with an OD lower than the OD value of the lowest sample concentration in the reference curve (0.033 vs approximately 0.250). Specificity to the antigen of interest was tested by spiking the WHO International Rabies Standard (0.310 IU as determined by *in vitro* assay) in the matrix. The ratio (expressed as percentage) of the experimental and theoretical antigen concentrations was 94%. A method is considered specific if this percentage lies in between 80% and 120%.

Table 1
Neutralization activity of mAbs D1-25 and WI 1112 against CVS11, PM and Flury LEP rabies virus strains.

mAb	Neutralizing activity (IU/μg mAb)		
	CVS11 strain	PM strain	Flury LEP strain
D1-25	0.079	0.16	0.10
WI 1112	3.22	2.66	2.72

Table 2
A: ELISA results on reduced/alkylated virus samples. B: ELISA results on BPL inactivated virus samples. Different batches were used for reduction/alkylation and BPL inactivation treatment which explains ELISA titer difference of control samples.

Sample treatment	ELISA titer (IU/ml)
A	
Reduction/alkylation control[a]	21.1
Reduced/alkylated sample	Not detectable
B	
BPL over-inactivation control[b]	30.7
BPL 1/4000 (v/v)	31.4
BPL 1/2000 (v/v)	26.8
BPL 1/1000 (v/v)	19.7
BPL 1/500 (v/v)	6.2

[a] Same sample treatment without reduction/alkylation.
[b] Same sample treatment without BPL inactivation.

Linearity was tested by conducting triplicate runs performed by 2 operators on different days. Each run involved the determination of 5 concentrations (3 diluted, 1 undiluted and 1 spiked vaccine samples). As illustrated in Fig. 1, the method was linear over the range 2.4–26.6 IU/mL with a corresponding coefficient of determination (R^2) of 1.0. Accuracy of measurement was observed over the same range as evidenced by an average recovery of 98% (95% confidence limits, 96–101%). This method can be considered precise since the 95% confidence interval of the average precision is multiplied or divided by 1.07 for 1 run. The ELISA was thus specific, linear, accurate, and precise and therefore valid to quantify G protein in PVRV-NG vaccine.

3.4. Consistency of vaccine production

Assessment of the consistency of vaccine production requires analytical tools that permit a rapid assessment of antigen during the production process as well as in the final product. For the PVRV-NG vaccine, this ELISA as well as the NIH test were performed in parallel at the final product stage. European Pharmacopoeia version 8.2 required 2.5 IU/dose, as determined by NIH test, in order to guarantee vaccine efficacy. In the current study, for all batches that were found to comply with the NIH potency specification (2.5 IU/dose), consistent results were obtained with the ELISA (Fig. 2).

3.5. Agreement of G-protein ELISA estimates with mouse potency tests

We assessed the relationship between the ELISA and the mouse

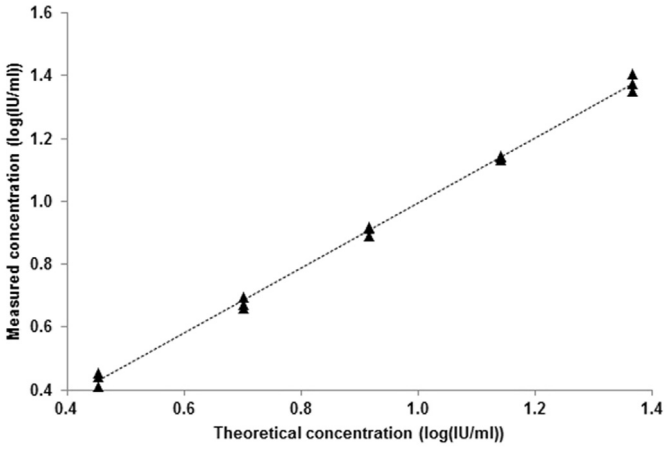

Fig. 1. Linearity of the ELISA. Plot of the log transformation of the theoretical against the experimentally determined G-protein concentrations.

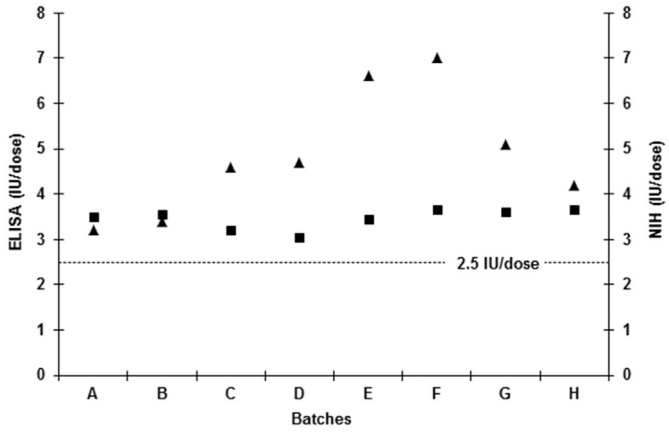

Fig. 2. Comparative analysis of different lots of rabies vaccine at the Filled Product step. The horizontal bar corresponds to the threshold for conformity. Triangle and right-hand y axis: NIH test; Square and left-hand y-axis: ELISA assay.

potency tests. NIH tests were performed for batches formulated at 1.1, 4.1 and 5.3 IU/dose as determined by ELISA. A dose-dependent variation of the NIH titer could be observed (Fig. 3). The batch at 1.1 ELISA IU/dose was tested twice by the NIH test, resulting in doses of 1.1 and 0.5 IU/dose. The batch at 4.1 ELISA IU/dose was tested 9 times by NIH, with titers varying between 3.7 and 8.3 IU/dose. The batch at 5.3 ELISA IU/dose was tested 8 times by NIH, with titers varying between 5.8 and 14.9 IU/dose. The ELISA was therefore able to detect subpotent batch obtained by formulation at different antigen concentration targets.

Secondly, subpotent samples were prepared by mixing potent lots with a heat-denatured lot. After heat inactivation no active G-protein could be detected by ELISA, or by the NIH test (Table 3). However, the heat-treated lot could protect 6 out of 16 mice injected with the lowest vaccine dilution (1/25), indicative of some

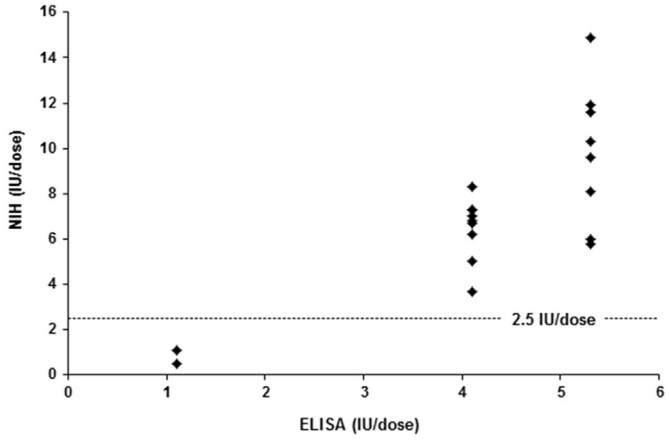

Fig. 3. Capacity of the NIH and ELISA tests to discriminate between potent and subpotent batches. Shown are data from the analysis of several batches formulated at different antigen content target: 1.1, 4.1 and 5.3 IU/dose (diamond shaped symbols) by the ELISA and the NIH test.

activity that could not be quantified by the NIH test or the ELISA. The intact lot had 3.3 ELISA IU/dose and 6.2 NIH IU/dose (Table 3). A mixture of equal volumes of a potent and a heat-denatured lot contained 1.4 ELISA IU/dose and 1.5 NIH IU/dose (Table 3), indicating that the ELISA can discriminate between potent and non-potent lots.

The agreement between NIH and ELISA tests was demonstrated by formulating three different bulks at concentrations of 3, 6 and 8 ELISA IU/dose. The 3 formulations of each bulk were tested in the same series of NIH tests. The results, representing duplicate experiments (individual batch values are reported in Table 4), showed a good agreement between the NIH test and the ELISA (Fig. 4).

3.6. Vaccine stability

To document the stability of the Filled Product, stability was assessed under ICH conditions, at the recommended storage temperature of 5 °C ± 3 °C with 4 different batches. Over a 24-month storage period, no trend was observed and the stability data complied with the specifications (Supp. Fig. 4). Additionally, the ELISA detected decreased levels of antigen after sample treatment at higher temperatures (Suppl. Fig. 5). The ELISA therefore meets the criteria for a stability-indicating assay based on its capacity to detect antigen degradation; the ELISA is also more powerful than the NIH test, a test with inherent variability.

4. Discussion

The present study was conducted as part of a global initiative to replace animal-based tests by alternative methods. Whereas the SRID test requires animal antiserum and has inherent difficulties in preparation and characterization, ELISA offers a rapid alternative based on the use of mAbs instead of animal antiserum. Here we developed an ELISA based on WI 1112 mAb for capture and D1-25 mAb for detection. We showed it can discriminate between native and modified forms of the rabies virus. This ELISA is able to quantify the rabies viral G-protein with high specificity, linearity, accuracy, and precision.

The NIH test has numerous limitations and disadvantages. The route of challenge differs from the natural infection route [23–25], which may lead to differences in the resultant immune response. The ELISA developed in the current study is based on the fact that the rabies viral G-protein is responsible for inducing neutralizing

Table 4
Individual batch values for duplicate NIH test.

Batch	NIH test 1	NIH test 2	Mean
S34-1	3.58	2.25	2.8
S34-3	5.1	7.4	5.9
S34-2	7.0	6.6	6.8
S31-1	3.58	3.48	3.5
S31-3	5.91	9.30	7.8
S31-2	8.16	15.38	10.2
S35-1	11.74	8.97	9.9
S35-3	15.68	8.59	10.5
S35-2	17.23	16.45	16.8

Table 3
Parallel analyses of the rabies vaccine lots at the filled batch stage by the NIH and ELISA tests.

Assay	Regular batch IU/dose	Heat-inactivated batch IU/dose	Equivolume mixture of regular and heat-inactivated batch IU/dose
ELISA	3.3	<0.1	1.4
NIH test	6.2	below quantification level	1.5

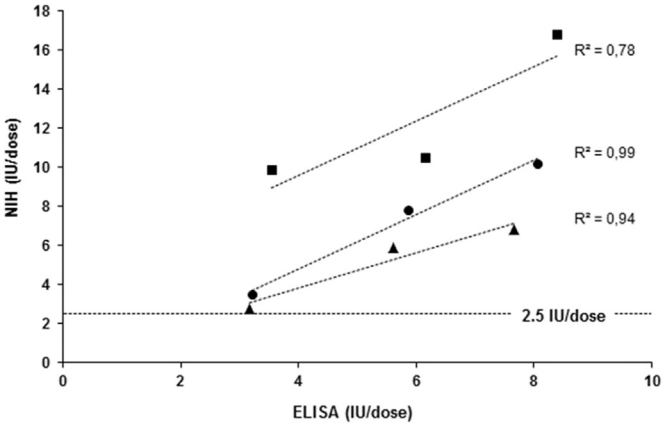

Fig. 4. Agreement between NIH test and ELISA assay. Shown are results of the analysis of three different bulks (square, round, triangle symbols) formulated each at 3 different targets (9 batches) by the ELISA and NIH tests. The 3 formulations of each bulk were tested in the same NIH series (3 samples in one NIH serie). Shown are the means of two NIH assays.

antibodies and that the structure of the G-protein influences its immunological properties. Indeed, approximately 83% of the neutralizing Abs are directed against the G-protein domain III [26] and this property depends on the three-dimensional structure of the G-protein [27]. The soluble or denatured G-protein is less immunogenic than its virion attached form [8,28,29].

Another feature underlying the usefulness of our ELISA resides in the low variability of the G-protein sequence among rabies strains [30]. Currently, rabies virus strains display a low variability of sites II and III, which should not interfere with mAb-G-protein interaction, indicating that the ELISA can detect a broad spectrum of rabies strains. A necessary requirement for potency tests is their capacity to distinguish between potent and subpotent batches. We show here that the ELISA is able to detect slight changes in the antigen concentration enabling the detection of batches that contain insufficient antigen amounts.

The ELISA is better suited to quantify antigen amounts than the NIH test, since the latter is characterized by a higher variability, due to its biological nature. The European Pharmacopoeia defined confidence limits of 25–400% [17], which implies that the NIH test is inappropriate for batch-to-batch consistency analyses. The higher precision of the ELISA permits its use for vaccine blending, as a release test or for the in-process monitoring.

Here, we undertook different approaches to modify the antigen and in each case the ELISA was able to detect G-protein alterations that could be related to potential process deviation such as BPL over-inactivation. Previous studies have advocated the replacement of the NIH test by the ELISA [12,31–33]; in-line with these studies, we observed a satisfactory agreement between the two tests in the current study (Fig. 4). Recently, an international workshop conducted by the NTP Interagency Centre for the Evaluation of Alternative Toxicological Methods, the Interagency Coordinating Committee on the Validation of Alternative Methods and their partners recommended a study showing a correlation between estimates of G-protein and potency [34]. As a consequence of this meeting, the European Partnership for Animal Alternatives (EPAA) set up a working group composed of Health Agencies and Experts to lead this NIH test replacement project. This ELISA was included in an EPAA pre-collaborative study, which has the objective to select the best antigen quantification assay based on its ability to recognize potent and subpotent lots from different origin and manufactured with different strains of rabies virus. It was considered as a good candidate and therefore selected for the European Directorate for the Quality of Medicines and Healthcare (EDQM) collaborative study [35] that will generate the scientific data towards the regulatory steps for the replacement of human rabies vaccine *in vivo* potency test by this ELISA in European pharmacopoeia. Our findings thus support efforts to achieve the goal of this replacement project.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biologicals.2017.02.002.

References

[1] Warrell MJ, Warrell DA. Rabies and other lyssavirus diseases. Lancet 2004;363:959–69.
[2] Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs. WHO TRS941 (Annexe II); 2007.
[3] Rabies vaccine for human use prepared in cell culture. eighth ed. Strasbourg. France: Council of Europe; 2015. Ph. Eur. monograph 0216.
[4] Barth R, Diderrich G, Weinmann E. NIH test, a problematic method for testing potency of inactivated rabies vaccine. Vaccine 1988;6:369–77.
[5] Isbrucker R, Levis R, Casey W, McFarland R, Schmitt M, Arciniega J. Alternative methods and strategies to reduce, refine, and replace animal use for human vaccine post-licensing safety testing: state of the science and future directions. Proc Vaccinol 2011;5:47–59.
[6] Nagarajan T, Reddy GS, Mohana Subramanian B, Rajalakshmi S, Thiagarajan D, Tordo N, et al. A simple immuno-capture ELISA to estimate rabies viral glycoprotein antigen in vaccine manufacture. Biologicals 2006;34:21–7.
[7] Gibert R, Alberti M, Poirier B, Jallet C, Tordo N, Morgeaux S. A relevant in vitro ELISA test in alternative to the in vivo NIH test for human rabies vaccine batch release. Vaccine 2013;31:6022–9.
[8] Gamoh K, Senda M, Itoh O, Muramatsu M, Hirayama N, Koike R, et al. Use of ELISA for in vitro potency test of rabies vaccines for animal use. Biologicals 1996;24:95–101.
[9] Luo TR, Minamoto N, Hishida M, Yamamoto K, Fujise T, Hiraga S, et al. Antigenic and functional analyses of glycoprotein of rabies virus using monoclonal antibodies. Microbiol Immunol 1998;42:187–93.
[10] Wiktor TJ, Gyorgy E, Schlumberger D, Sokol F, Koprowski H. Antigenic properties of rabies virus components. J Immunol 1973;110:269–76.
[11] Perrin P, Thibodeau L, Sureau P. Rabies immunosome (subunit vaccine) structure and immunogenicity. Pre- and post-exposure protection studies. Vaccine 1985;3:325–32.
[12] Lafon M, Perrin P, Versmisse P, Sureau P. Use of a monoclonal antibody for quantitation of rabies vaccine glycoprotein by enzyme immunoassay. J Biol Stand 1985;13:295–301.
[13] Majer M, Herrmann A, Hilfenhaus J, Mauler R, Lehmann HG, Hennessen W, et al. A comparison of the Pasteur and Pitman-Moore strains of rabies virus for the production of rabies vaccine in human diploid cells. J Biol Stand 1977;5:249–56.
[14] Schumacher CL, Dietzschold B, Ertl HC, Niu HS, Rupprecht CE, Koprowski H. Use of mouse anti-rabies monoclonal antibodies in postexposure treatment of rabies. J Clin Invest 1989 Sep;84(3):971–5.
[15] Müller T, Dietzschold B, Ertl H, Fooks AR, Freuling C, Fehlner-Gardiner C, et al. Development of a mouse monoclonal antibody cocktail for post-exposure rabies prophylaxis in humans. PLoS Negl Trop Dis 2009 Nov 3;3(11):e542.
[16] Elia G. Protein biotinylation. Current protocols in protein science/editorial board. John E Coligan [et al]. 2010 [Chapter 3]:Unit 3 6.
[17] Statistical analysis of results of biological assays and tests (parallel line analysis described in section 3.2). European pharmacopeia monograph 5.3 (01/2008). in Section 3.22008.
[18] Jallet C, Jacob Y, Bahloul C, Drings A, Desmezieres E, Tordo N, et al. Chimeric lyssavirus glycoproteins with increased immunological potential. J Virol 1999;73:225–33.
[19] Dietzschold B, Gore M, Casali P, Ueki Y, Rupprecht CE, Notkins AL, et al. Biological characterization of human monoclonal antibodies to rabies virus. J Virol 1990;64:3087–90.
[20] Bakker AB, Marissen WE, Kramer RA, Rice AB, Weldon WC, Niezgoda M, et al. Novel human monoclonal antibody combination effectively neutralizing natural rabies virus variants and individual in vitro escape mutants. J Virol 2005;79:9062–8.

[21] Toinon A, Greco F, Moreno N, Nicolai M-C, Guinet-Morlot F, Manin C, et al. Study of rabies virus by differential scanning calorimetry. Biochem Biophys Rep 2015;4:329–36.
[22] International conference on harmonisation of technical requirement for registration of Pharmaceuticals for human use. ICH harmonised tripartite guideline. Validation of analytical procedures: text and methodology Q2(R1). Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.
[23] Wunderli PS, Dreesen DW, Miller TJ, Baer GM. Effects of vaccine route and dosage on protection from rabies after intracerebral challenge in mice. Am J Vet Res 2003;64:491–8.
[24] Kramer B, Schildger H, Behrensdorf-Nicol HA, Hanschmann KM, Duchow K. The rapid fluorescent focus inhibition test is a suitable method for batch potency testing of inactivated rabies vaccines. Biologicals 2009;37:119–26.
[25] Wunderli PS, Shaddock JH, Schmid DS, Miller TJ, Baer GM. The protective role of humoral neutralizing antibody in the NIH potency test for rabies vaccines. Vaccine 1991;9:638–42.
[26] Kramer RA, Marissen WE, Goudsmit J, Visser TJ, Clijsters-Van der Horst M, Bakker AQ, et al. The human antibody repertoire specific for rabies virus glycoprotein as selected from immune libraries. Eur J Immunol 2005;35:2131–45.
[27] Bunschoten H, Gore M, Claassen IJ, Uytdehaag FG, Dietzschold B, Wunner WH, et al. Characterization of a new virus-neutralizing epitope that denotes a sequential determinant on the rabies virus glycoprotein. J Gen Virol 1989;70:291–8.
[28] Dietzschold B, Wiktor TJ, Wunner WH, Varrichio A. Chemical and immunological analysis of the rabies soluble glycoprotein. Virology 1983;124:330–7.
[29] Piza AT, Pieri KM, Lusa GM, Caporale GM, Terreran MT, Machado LA, et al. Effect of the contents and form of rabies glycoprotein on the potency of rabies vaccination in cattle. Mem Inst Oswaldo Cruz 2002;97:265–8.
[30] Holmes EC, Woelk CH, Kassis R, Bourhy H. Genetic constraints and the adaptive evolution of rabies virus in nature. Virology 2002;292:247–57.
[31] Thraenhart O, Ramakrishnan K. Standardization of an enzyme immunoassay for the in vitro potency assay of inactivated tissue culture rabies vaccines: determination of the rabies virus glycoprotein with polyclonal antisera. J Biol Stand 1989;17:291–309.
[32] Perrin P, Morgeaux S, Sureau P. In vitro rabies vaccine potency appraisal by ELISA: advantages of the immunocapture method with a neutralizing anti-glycoprotein monoclonal antibody. Biologicals 1990;18:321–30.
[33] Rooijakkers EJ, Uittenbogaard JP, Groen J, Osterhaus AD. Rabies vaccine potency control: comparison of ELISA systems for antigenicity testing. J Virol Methods 1996;58:111–9.
[34] Stokes W, McFarland R, Kulpa-Eddy J, Gatewood D, Levis R, Halder M, et al. Report on the international workshop on alternative methods for human and veterinary rabies vaccine testing: state of the science and planning the way forward. Biologicals 2012;40:369–81.
[35] Morgeaux S, Poirier B, Ragan CI, Wilkinson D, Arabin U, Guinet-Morlot F, et al. Replacement of in vivo human rabies vaccine potency testing by in vitro glycoprotein quantification using ELISA - results of an international collaborative study. Vaccine 2017 Feb 7;35(6):966–97.