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Verification of the rabies virus glycoprotein lower limit of immunogenicity by serological assay

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ABSTRACT

Rabies lethality is close to 100% and annually 15 million people receive post-exposure prophylaxis. Testing for vaccines against this zoonosis should ensure its quality. A standardized test by the National Institutes of Health (NIH) test, based on mice immunization and challenge, has been used to determine the potency of vaccine lots. It has several disadvantages, the main one being its significant variability. Several in vitro methods like an Enzyme-linked immunosorbent assay (ELISA) have been proposed based on the quality and quantity of glycoprotein (Glptn) of rabies virus, but may also present limitations such as low sensitivity, instability and imprecision. The estimate of immunogenicity based on neutralizing antibody titer (Nab) evaluated by a serological test (ST) such as the Modified Rapid Fluorescent Focus Inhibition Test (mRFFIT), is not yet efectively applied for human vaccine. Nevertheless, a Nab concentration can be used as a predictor of clinical efficacy of this product in vaccinated humans, so, that can be applied in estimating the vaccine potency. The aim of this study was to verify the lower limit of immunogenicity of the viral Glptn content in mice using mRFFIT. The lower Glptn content by ELISA able to induce Nab response was determined. The results were correlated and demonstrated that ST was able to determine the Glptn immunogenicity lower limit. Our findings suggest that a test based on rabies Nabs may represent an additional alternative for the evaluation of rabies vaccines.

1. Introduction

Rabies is an almost 100% lethal zoonosis transmitted to humans by the inoculation of rabies viruses (RABV), which are members of the *Rabies lyssavirus*genus, which belongs to the Rhabdoviridae family and Mononegavirales order (Rupprecht et al., 2017; ICTV, 2011). Almost all vaccines are based on the functional aspects of G glycoprotein (Glptn), the major antigen of the virus that confers immunity against lethal infection. Antibodies exert their effect by extracellular virus neutralization, complement-mediated lysis of infected cells, and antibody-dependent cytotoxicity (Mattos et al., 2001). The antigenicity of Glptn is conferred by two dominant antigenic sites (II and III), composed of 20 amino acids out of a total of 505 (Dietzschold et al., 1990; Benmansour et al., 1991).

Human rabies vaccines have their potency assessed by the National Institute of Health (NIH) test (Seligmann, 1966) and must have 2.5 IU/dose as minimum potency (Brasil, 2010a). Batches are submitted to viral identification, sterility and viral titers. In addition, the viral strain must have demonstrated adequate immunogenicity for humans (Brasil, 2010a; Council of Europe, 2015).

The first potency test in mice described was the Habel test (Habel, 1966), which was originally developed in 1944, and it was progressively replaced by NIH test. This is an in vivo method consisting of intraperitoneal (ip) immunization of mice groups, followed by an intracerebral (ic) challenge with standard virus (CVS). The animals are monitored for 14 days post challenge and the relative potency is calculated by comparing their mean effective dose or 50% (ED50) with the ED50 of a reference vaccine (Wilbur and Aubert, 1996).

In the last decades, several antigen and serological quantification models have been developed as potential alternatives for the activity assay. An example of this is the serologic assay based on fluorescent focus inhibition test (RFFIT) for inactivated veterinary vacine (Krämer et al., 2009, 2010). The European Pharmacopoeia proposes two methods for determining the potency of this same vaccine: (i) the

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classical challenge test and (ii) the titration of neutralizing antibodies (Nab) induced after mice vaccination, revealed by RFFIT (Council of Europe, 2013). Although the method based on Nab is faster and less painful for animals, it is still not widely used and there are only few data about the agreement with the classical method (Fitzgerald et al., 1978; Krämer et al., 2009). Another disadvantage is that this test is not able to determine the potency of a vaccine. In Brazil, the challenge method and determination of immunogenic activity are applied to rabies vaccine (Brasil, 2010a). For inactivated veterinary vaccines, the Agriculture Ministry currently uses the NIH test (Brasil, 1988).

Antigen quantification models anticipate total animal replacement and measure the amount of antigen or immunogen of the vaccine. These include the single radial immunodiffusion assay (Ferguson et al., 1996), which was accepted in the batch release (Bruckner et al., 2003), the antibody binding test (Arko et al., 1973) and various Enzyme-Linked Immunosorbent Assay (ELISA) protocols (Perrin et al., 1990; Rooijakkers et al., 1996; Gamoh et al., 1996). Among these, there is an ELISA used by the National Control Laboratory of the National Agency for the Safety of Medicines and Health Products of France (Agence National de Sécurité du Medicament et des Produits de Santé) to quantify the viral Glptn in vacines without adjuvant (Stokes et al., 2012). Several ELISA assays for rabies vaccine potency have been proposed as alternatives to the NIH test (Perrin et al., 1990; Rooijakkers et al., 1996; Gamoh et al., 1996). Others are still under development, such as ELISA for viral G-protein in human anti-rabies vaccine without adjuvant (Morgeaux et al., 2017; Chabaud-Riou et al., 2017). These methods for rabies G-protein estimation using monoclonal antibodies directed against site III (Nagarajan et al., 2006; Gibert et al., 2013) or site II (Gamoh et al., 1996; Luo et al., 1998) of this protein.

However, there are still several scientific suspicions for the application of serological or antigen quantification models that lead to hesitations regarding the acceptance of these methods for regulatory purposes (Stokes et al., 2011; Schiffelers et al., 2014). Although it has already been shown that induction of Nab by a vaccine is a function of the antigen concentration present in a given vaccine dilution in the NIH test (Diaz et al., 1990), there are doubts whether the mice are suitable models and could predict exactly the vaccine effect in man, or the cell-mediated response. It is also possible that the NIH test has limitations and can not accurately predict the effectiveness of rabies vaccines in humans. This allows us to infer that with this type of test it is not possible to predict vaccine efficacy in humans or in a cell-mediated response, but it is an adequate test to verify the consistency of production by applying trend analysis. The present study aimed to evaluate the lowest Glptn content (determined by D1 ELISA kit – Pasteur Institute, France) capable of inducing a suitable Nab response (> 0.5 IU/ mL) in mice, using modified RFFIT adapted for 96-well microplate (mRFFIT).

2. Animals, materials and methods

Tests were carried out in order to assess the serological assay sensitivity and establish the correlation between immunogenicity and viral Glptn content to determine the lower limit of immunogenicity to viral Glptn.

2.1. Laboratory animals

Swiss Webster mice (outbred strain), male and female, about 3–4 weeks old, weighing 10–15 g, were obtained from FIOCRUZ / Institute of Biomodel Science and Technology (ICTB - Brazil). Animals were separated by genus, housed in appropriate cages with water and food ad libitum, kept under controlled temperature and humidity (22 ± 1 °C, about 50% relative humidity and 12: 12 h photoperiod).

For the NIH test, 48 mice were used per vaccine, 16 animals for each of 3 dilutions and 3 groups of 10 mice for virus titration. For the Serological potency test (SPT), 15 mice were used per vaccine, 5 animals for each of 3 dilutions, both for the samples and reference vaccine (Moreira et al., 2019). A total of 1289 animals were used in this research in 5 NIH tests and 7 SPT tests. This study was approved by the FIOCRUZ Institutional Animal Care And Use Committee (LW-17/16.).

Table 1
Rabies Vaccine Samples.

Sample	Potency (IU/ dose)	
	INCQS	Producer
Vac 1	19.4	14.4
Vac 2	8.1	4.8
Vac 3	nt	6.8
BR014	9.0	14.9
Vac 5	nt	17.6
Vac 6	nt	4.4
Vac 7	12.3	na

Potency determined by NIH test. nt – not tested. na - not applicable.

2.2. Vaccine samples

2.2.1. Test vaccines

Seven batches of rabies vaccine that were approved in potency test in the laboratory routine were selected. Rabies vaccines are presented as freeze-dried with 0.5 mL diluent in one-dose vials. They are produced in Vero cells using the Wistar PM/WI38 1503-3 M strain of RABV, inactivated by betapropriolactone and purified. The minimum potency required is 2.5 IU/ dose (Table 1).

2.2.2. Reference vaccine

The Brazilian National Rabies Reference Vaccine BR013 (Refvac) is a cell culture vaccine produced in Vero cells, using the RABV PV strain, inactivated by betapropriolactone and lyophilized in multi-dose vials. The lot was manufactured in 2005. The BR013 was certified according to WHO recommendations (WHO, 2006), by the Instituto Nacional de Controle de Qualidade em Saúde (INCQS), FIOCRUZ, against the 6th International Standard provided by the National Institute of Biological Standards and Control (NIBSC, UK), with the attributed Glptn content of 9.53 IU/mL.

The Pasteur Rabies Reference Vaccine (Pasvac) is provided with D1 ELISA kits – Pasteur Institute (France) with the attributed concentration of 1000 ng/mL of RV Glptn.

2.3. NIH test

The NIH test consisted in two ip immunizations (0 and 7 day), with 0.5 mL of the dilutions (Vac 1–3 and 5–7 - 1/25, 1/125 and 1/625) of the test vaccines or Refvac (1/50, 1/250 and 1/1250). The challenge was performed on day 14 by ic route with about 32 LD50/ 0.03 mL of CVS. Simultaneously the challenge virus was titrated. The animals were observed for 14 days and humane endpoints were applied, when they developed signs of rabies. The acceptance criteria adopted were those described in the Brazilian Pharmacopoeia (Brasil, 2010a).

2.4. Serological potency test for rabies vaccines of human use (SPT)

In order to standardize the SPT, for the determination of Nab titers against rabies, sera from vaccinated mice were evaluated by mRFFIT. The vaccination schedule consisted in two ip immunizations (0 and 7 day), with 0.5 mL of the dilutions (Vac 1, 2 and 3 - 1/25, 1/125 and 1/625; Vac 3 - 1/250, 1/1250 and 1/6250; Vac 5 - 1/1100, 1/5500 and 1/27,500; Vac 6 – 1/200, 1/1000 and 1/5000) of the test vaccines or Refvac (1/50, 1/250 and 1/1250). On day 14 after immunization the mice were bleed by cardiac puncture under anesthesia. The average volume of blood obtained by animal was about 1.5 ml. The blood

samples were centrifuged after retraction of the clot at 200 g for 15 ± 1 min and sera transferred to sterile cryovials. Sera were inactivated at 56 ± 1 °C for 30 ± 1 min and kept at -20 ± 1 °C until be tested.

Fifty µl of test sera were serially two-fold diluted from 1/4 to 1/32 and the working virus suspension was diluted to contain 200 focus-forming dose 50% (FFD_{50}). Plates were incubated at 36.5 ± 0.5 °C in a humidified incubator with 5% CO_2 for 90 min. After this time the BHK-21 cell suspension (3.5×10^5 cells/ ml) was added and the plates were incubated for 22 h. The plates were washed with PBS, fixed with acetone (80%) and incubated with the comertial rabies nucleocapsid conjugate (Bio-RAD, France) at 37 °C for 30 min. After washing and drying, the plates were analyzed under UV microscope to determine the number of positive fields per wells (Brasil, 2010b; Moura et al., 2008). Mice sera that completely neutralized the 1/32 dilution were retested in 1/64 to 1/512 dilutions. The Nab titer was calculated using the probit analysis in CombiStats 5.0 (EDQM) and expressed in IU/ ml.

The relative potencies of vaccines were determined in SPT by calculation approach that uses the cut-off ≥0.5 IU/ ml as an indicative of seroconversion titer (Fitzgerald et al., 1978; Wunderli et al., 1991; WHO, 2005; World Organisation for Animal Health - OIE, 2008). The minimum requirement of vaccine potency adopted for the SPT was ≥2.5 IU/ vial, as in the NIH test. Probit analysis is a type of regression used to analyze binomial response variables. It transforms the sigmoid dose-response curve to a straight line which can be further analyzed by regression either through least squares or through maximum likelihood (Finney, 1971). The SPT protocol was developed and pre-validate, preliminary, as a direct adaptation of NIH test (Moreira et al., 2019).

2.5. Enzyme-linked immunosorbent assay (ELISA)

To evaluate the Glptn content, the antigen was determined in the vaccine samples by the Laboratory of Analytical Development and Stability from Butantan Institute (São Paulo, Brazil), using the D1 kit from the Pasteur Institute (Paris, France). The Glptn content was determined by the collaborating laboratory that used its own internal ELISA protocol applying murine D1 monoclonal antibody (mAb-D1) (Jallet et al., 1999) for both plate coating and virus detection (Perrin et al., 1996). The mAb-D1 IgG1 isotype recognizes the antigenic site III (involved in virus Nab induction) of native Glptn. The vaccines Glptn content was quantified against two reference standards: Institute Pasteur D1 kit standard (1000 ng/ mL) and INCQS BR013 vaccine (9.53 IU/ mL) (Fournier-Caruana et al., 2003). The Glptn concentrations in each of the dilutions were calculated from extrapolations obtained from the total Glptn determined from undiluted formulations considering the dilution factor.

2.6. Verification of the lower limit of immunogenicity of viral glycoprotein

In order to estimate the lowest antigen concentration inducing adequate immune response (≥0.5 IU/ ml), serum samples of mice immunized with seven vaccines of known potency and Glptn content were obtained to achieve minimum antigen levels and to exhaust immune response.

In order to exhaust immune response, three of the vaccine batches were diluted from the ED50 determined at NIH test to get three fivefold serial dilutions (Vac 3, 5 and 6). In Group 1 the vaccine was prepared at the dilution of ED50, in Group 2 it was adjusted to ED50/ 5 per dose and in the third group diluted ED50/ 25. Three groups of 10 animals were immunized with two doses (days 0 and 7), ip route with each dilution obtained. In the other four batches (Vac 1, 2, BR014 and 7) the procedures for standardizing the SPT were performed.

After immunizations, blood samples were collected on day 14 and the sera titrated for determination of the Nab titers by mRFFIT. A total of 45 dilutions were tested, yielding 223 (225-2 losses) individual titer results from mice sera and the means were calculated for each dilution (n = 45). The mean titers of Nab were expressed in relation to the dilutions nominal Glptn content in ng/ ml and IU/ ml. The Glptn concentration versus responses curves were calculated in logarithm transformed results by simple linear regression. The data were rearranged in increase order of the mean Glptn titer and verified the starting dilution from which there was induction of adequate immune response.

2.7. Statistical analysis

The results were evaluated by calculating the degree of correlation by simple linear regression and Pearson correlation coefficient in order to determine the degree of association between the data and its significance. The lowest concentration was determined by visual inspection of the results increase reordered as the lowest dilution, expressed in Glptn concentration in ng/ ml and in IU/ ml from which a suitable Nab titer was obtained, based on the cutoff addopted. For statistical analysis, Microsoft® Excel, GraphPrism®, MediCalc® and CombiStats® Software were used. When necessary, the potency results were submitted to a logarithmic transformation to improve the normal distribution.

3. Results

The in vitro rabies Nab titers of 344 vaccinated mice sera samples were examined by mRFFIT and the potencies were determined in SPT (Table 2).

Table 3 shows the Glptn content of 7 vaccine lots obtained by Institute Pasteur ELISA-D1 kit. It also includes the Glptn concentration declared by the producer in the vaccine production protocol and the potency of the vaccines in NIH test and SPT.

In the evaluation of the Glptn content against the Refvac, compared to the means of Nab titers, there was a seroconversion from 0.0138 IU/ ml of Glptn. After the analysis of the individual Nab titers results, non-responders were identified and these results were removed from the calculation of the means (Vac 1 - one mouse, 1/ 125, Vac 3 - two mice, 1/ 1250 and 1/ 6250). One exception (Vac 4, 1/1250) was detected, where seroconvertion (0.90 IU /ml) with a concentration of 0.00277 IU /ml Glptn occurred (Table 4).

In the Glptn content determined in ng/ ml against the Pasvac, seroconversion occurred from the concentration of 0.388 ng/ ml Glptn. The BR014 (1/ 1250) also presented seroconvertion with a lower content (0.078 ng/ ml). The same procedure for the removal of non-responders was adopted in the calculation of the mean values of Nab titers (Table 5).

A linear regression was performed to verify if there was an association between the log values of the Nab titers of the mice obtained by the mRFFIT and the results of the Glptn content against Refvac (IU/ ml). It was observed that the relationship between these two results was significantly correlated ($r = 0.758$; $p < 0.0001$) (Fig. 1A). The log values of the Nab titers mean were also compared by linear regression with the Glptn content against Pasvac (ng/ ml). Once again, the results showed a significant correlation ($r = 0.758$, $p < 0.0001$) (Fig. 1B) (Table 5).

The same procedure was applied to the log values of the mean Nab titers of the BR014. Linear regression demonstrated that the results were correlated with Glptn content (IU/ ml) with the Refvac as standard. The line equation was $Y = 0.4429X + 0.7701$ ($r = 0.913$, $p = 0.2676$) (Fig. 2A). Although the value of r was high, indicating a strong linear correlation of the data, the p-value (> 0.05) showed no statistical significance in the correlation probably due to the small number of data. The linear regression of the Nab titers logarithms of the BR014 against Glptn content (ng/ ml) compared to the Pasvac, also presented the correlated results. The line equation was $Y = 0.3714X + 0.06562$ ($r = 0.921$; $p = 0.2542$) (Fig. 2B). Although the value of r was high too, showing a strong linear correlation of the data, the p-value (> 0.05) indicated that there was no statistical significance in the

Table 2
Dilution of rabies vaccines, glycoprotein content against Refvac (IU/ ml) and Pasvac (ng/ ml) by ELISA and means of neutralizing antibody titers (IU/ ml) by mRFFIT in the SPT.

Sample	Dilution	ELISA		SPT	ELISA		SPT	ELISA		SPT	ELISA		SPT	ELISA		SPT
		IU/ml	ng/ml	Nab IU/ml	IU/ml	ng/ml	Nab IU/ml	IU/ml	ng/ml	Nab IU/ ml	IU/ml	ng/ml	Nab IU/ ml	IU/ml	ng/ml	Nab IU/ ml
BR014	1/50	0.06929	1.958	0.87	0.06929	1.958	1.03	0.06929	1.958	1.00	0.06929	1.958	1.47	0.06920	1.958	2.03
	1/250	0.01386	0.392	1.67	0.01386	0.392	0.57	0.01386	0.392	1.00	0.01386	0.392	1.03	0.01384	0.392	1.26
	1/1250	0.00277	0.078	0.07	0.00277	0.078	0.25	0.00277	0.078	0.09	0.00277	0.078	0.90	0.00280	0.078	0.23
Vac 1	1/25	0.18246	4.060	0.80	0.18246	4.060	0.81									
	1/125	0.03649	0.812	0.55*	0.03649	0.812	0.64									
	1/625	0.00730	0.162	0.28	0.00730	0.162	0.20									
Vac 2	1/25	0.20318	4.257	0.89												
	1/125	0.04064	0.851	0.86												
	1/625	0.00813	0.170	0.28												
Vac 3	1/25	0.26499	3.880	2.06												
	1/125	0.05300	0.776	0.74												
	1/625	0.01060	0.155	0.28												
	1/250	0.02650	0.388	1.36	0.02650	0.388	1.11									
	1/1250	0.00530	0.078	0.25	0.00530	0.078	0.25									
	1/6250	0.00106	0.016	0.24**	0.00106	0.016	0.29**									
Vac 5	1/1100	0.00300	0.080	0.19	0.00300	0.080	0.25									
	1/5500	0.00060	0.016	0.11	0.00060	0.016	0.12									
	1/27,500	0.00012	0.003	0.15	0.00012	0.003	0.26									
Vac 6	1/200	0.03377	0.662	1.18	0.03377	0.662	1.63									
	1/1000	0.00675	0.132	0.20	0.00675	0.132	0.18									
	1/5000	0.00135	0.026	0.35	0.00135	0.026	0.12									

Nab mean after removal *one and **two animals.

Table 3
Rabies vaccines Glycoprotein content and potency in the NIH and serological tests.

Sample	Glptn			Potency (IU/ dose)	
	(ng/ ml)[1]	(IU/ ml)[2]	(IU/ ml)[3]	NIH	SPT[4]
Vac 1	101.50	4.56	6.6	19.44	6.39
Vac 2	106.43	5.08	6.6	8.13	6.62
Vac 3	96.99	6.62	7.4	6.8*	9.46
BR014	97.91	3.46	4.0	9.0	nd
Vac 5	88.07	3.30	8.2	17.6*	nd
Vac 6	132.38	6.75	8.8	4.4*	nd
Vac 7	107.08	4.45	na	12.3	nd

[1] Glycoprotein content determined by ELISA applying murine D1 antibody for plate coating and virus detection against the Pasvac (1000 ng/ ml) and.
[2] Refvac (9.53 IU/ ml).
[3] Glptn declared by the producer.
[4] Determination of relative potency by SPT.
* Potency determined by the producer. nd - not done. na - not applicable.

Table 4
Glycoprotein content of rabies vaccines (IU/ ml) against Refvac and means of neutralizing antibody titers of mice.

Glptn[1]	Nab[2]	Glptn	Nab	Glptn	Nab
0.00012	0.15	0.00530	0.25	0.03377	1.18
0.00012	0.26	0.00530	0.25	0.03377	1.63
0.00060	0.11	0.00675	0.20	0.03649	0.55**
0.00060	0.12	0.00675	0.18	0.03649	0.64
0.00106	0.24*	0.00730	0.28	0.04064	0.86
0.00106	0.29*	0.00730	0.20	0.05300	0.74
0.00135	0.35	0.00813	0.28	0.06920	2.03
0.00135	0.12	0.01060	0.28	0.06929	0.87
0.00277	0.23	0.01384	1.26	0.06929	1.03
0.00277	0.07	0.01386	1.67	0.06929	1.00
0.00277	0.25	0.01386	0.57	0.06929	1.47
0.00277	0.09	0.01386	1.00	0.18246	0.80
0.00277	0.90	0.01386	1.03	0.18246	0.81
0.00300	0.19	0.02650	1.36	0.20318	0.89
0.00300	0.25	0.02650	1.11	0.26499	2.06

[1] Glycoprotein content (IU/ ml) determined by ELISA-D1 against Refvac.
[2] Means of Nab titers (IU/ ml) determined in mRFFIT per vaccine dilution. Hatched cells indicate seroconversion ≥0.5 IU/ ml.
* Nab mean after removal of two animals.
** Mean after removal one mouse. n = 45.

correlation may be due to the small number of data.

After this verification, it was established that the minimum concentration of Glptn capable of inducing a satisfactory immune response (≥0.5 IU/ ml) for the vaccines of this study, and to guarantee efficacy, was 0.0138 IU/ ml or 0.388 ng/ ml.

Intra- and inter-assay variances of the Nab titers logarithms were calculated by dilution. So, it was obtained the intra-assay gCV% of 31.99 (SPT 01/17) and 31.65 (SPT 02/17) and inter-assays gCV% of 31.82%.

4. Discussion

For better understand of the sensitivity of serological assays based on viral neutralization revealed in cell culture, the study for determining the lower limit of immunogenicity to Glptn was carried out. In this study it was calculated the correlation between the Glptn content, measured by ELISA-D1 kit and the title of Nab, assessed by mRFFIT.

The seven batches of vaccines used in this study contained different Glptn concentrations, ranging from 3.3 to 6.75 IU/ ml or 88.07 to 132.38 ng/ ml. The mean of the Nab titers were paired with the Glptn content, the concentration of effective seroconversion (0.00138 IU/ ml, 0.388 ng/ ml of Glptn) was identified, and the results were directly correlated by linear regression.

The correlation coefficients between Glptn content and Nab titers mean were considered suitable (r = 0.758), higher than that observed by other research groups that found a weak correlation (Smith et al., 2013). The same was observed for BR014 which the results were also correlated (r = 0.913 and 0.921). These data showed a positive and strong correlation between the antibody response and the antigen amount in the vaccine such as reveled in the study about furunculosis vaccines in Atlantic salmon, whose find Pearson's Correlation of r = 0.82 (Romstad et al., 2013). The same results of positive correlation between the antibody response of H9N2 inactivated vaccine formulas in

Table 5
Glycoprotein content of rabies vaccines (ng/ ml) against Pasvac and means of neutralizing antibody titers of mice.

Glptn[1]	Nab[2]	Glptn	Nab	Glptn	Nab
0.003	0.15	0.080	0.19	0.662	1.18
0.003	0.26	0.080	0.25	0.662	1.63
0.016	0.24*	0.016	0.20	0.776	0.74
0.016	0.29*	0.003	0.18	0.812	0.55**
0.016	0.11	0.155	0.28	0.812	0.64
0.016	0.12	0.162	0.28	0.851	0.86
0.003	0.35	0.162	0.20	1.958	0.87
0.001	0.12	0.170	0.28	1.958	1.03
0.078	0.25	0.388	1.36	1.958	1.00
0.078	0.25	0.388	1.11	1.958	1.47
0.078	0.07	0.392	1.67	1.958	2.03
0.078	0.25	0.392	0.57	3.880	2.06
0.078	0.09	0.392	1.00	4.060	0.80
0.078	0.90	0.392	1.03	4.060	0.81
0.078	0.23	0.392	1.26	4.257	0.89

[1] Glycoprotein content (ng/ ml) determined by ELISA-D1 against Pasvac.
[2] Means of Nab titers (IU/ ml) by mRFFIT per vaccine dilution. Crosshatched cells indicate seroconversion ≥0.5 IU/ ml.
* Nab mean after removal of two animals.
** Mean after removal one mouse. n = 45.

both commercial broilers and specific-pathogen-free (SPF)-chickens were also reported (Kilany et al., 2016). The serological response of SPF-chickens was measured after immunization with inactivated Newcastle disease vaccines and the content of the vaccines was highly correlated with the antibody titers (r = 0.87 and 0.97), similar to our findings (Maas et al., 2003). In a clinical trial involving adult volunteers where anti-rabies vaccines were tested (Ferguson and Schild, 1982), and in another study with cattle (Schumacher et al., 1989; Piza et al., 2002), antibody responses showed a correlation between the antigenic content of the vaccine and the Nab titer similar to that observed in mice in our experiments.

In the study of Romstad et al. (2013), there is also a close correlation between the antibody response and protection against challenge. This is another similarity to our study because we believe that low titers of Nab would not protect the animals from an ic challenge as previously described (Fitzgerald et al., 1978; Wunderli et al., 1991), and here we observe that lower antigen content worse the seroconversion is.

Classically, the efficacy of immunobiological products for rabies is demonstrated by methods that use ic challenge as indicative of adequate immune response (Brazil, 2010a, Council of Europe, 2013, Council of Europe, 2015). Studies have shown that the survival rate of NIH is correlated with Nab titers in mice (Fitzgerald et al., 1978; Wunderli et al., 1991). Therefore, the Nab titer ≥0.5 IU/ ml was adopted as the minimum dose conferring protection in mice. Since a correlation was found between the Nab titer and the Glptn content, it can be inferred that the serological assay described here may be a candidate to replace the NIH. Nevertheless, an approach using ic challenge and the assessment of the Glptn lower limit of immunogenicity, combined with the serologic evaluation, could be useful in the search for improvements in SPT for the purpose of future replacement of NIH.

Future validation studies should include potent, sub-potent, and borderline samples classified by the NIH to access the sensitivity of the SPT and to verify if the method is able to distinguish between regular and sub-potent batches of vaccines. In this way it will be possible to evaluate the methodology and propose improvements for the test.

An advantage of the SPT is directly measuring the Nab title and vaccine immunogenicity. This fact may be convenient in the search for a replacement of the NIH test. The mRFFIT was as effective as NIH test in estimating the ability of a vaccine batch to induce protection against RABV infection, as recognized by other researchers (Gibert et al., 2013). This same hypothesis also has already been raised previously using other assays to assess immunogenicity by quantifying Glptn (Smith et al., 2013). But we agree that, as other authors claim, in vitro methods measure binding antibodies, whereas in vivo tests, such as mRFFIT, measure Nab and directly reveal humoral immunity, being safer.

Today, ELISA and simple radial immunodiffusion are recommended to determine antigen content in the formulation of human vaccine batches, but the NIH test is maintained in the potency assessment (WHO, 2007; Council of Europe, 2015). However, it has been demonstrated that induction of Nab by a vaccine is a function of the concentration of antigen present in a given vaccine dilution in NIH test (Diaz et al., 1990), as noted here. This allows to infer that this type of test is adequate to verify the consistency of production being indicative of the efficacy of the vaccine (WHO, 2007; Council of Europe, 2013). In addition to the considerable reduction of the animal's number, combining this approach with GMP and other quality systems, together with the statistical analysis at each stage of the production process, serological assays in the consistency approach can be applied in batch release. Thus, it will be possible to obtain quality and reduce the in vivo tests on finished products as indicated in other similar studies (Kreeftenberg, 2002; Arciniega and Sirota, 2012).

The rabies vaccine is the only pharmaceutical product in which the minimum dose has not been defined, or as already pointed out by other research (Dodet, 2011), how much active component should be injected into the patient. This becomes a problem for the control of the vaccine schemes and for the evaluation of patient's seroconversion. WHO recommends the same potency (2.5 IU) for all cell culture vaccines, and these can be applied by intra-muscular or intradermal routes, but dose volumes are fixed although the vaccine batch potency. For this reason, the results presented here may also contribute in this field.

In conclusion, the lowest Glptn content determined by ELISA capable of inducing a suitable Nab response in mice was found. Positive, significant and linear correlation was found between the Glptn content and Nab titer demonstrating the potential of serological assay mRFFIT-based for checking the compliance of the vaccine lots to specifications and being a candidate for future research and validation. Considerable refinement and reduction in animal use are expected, and the method

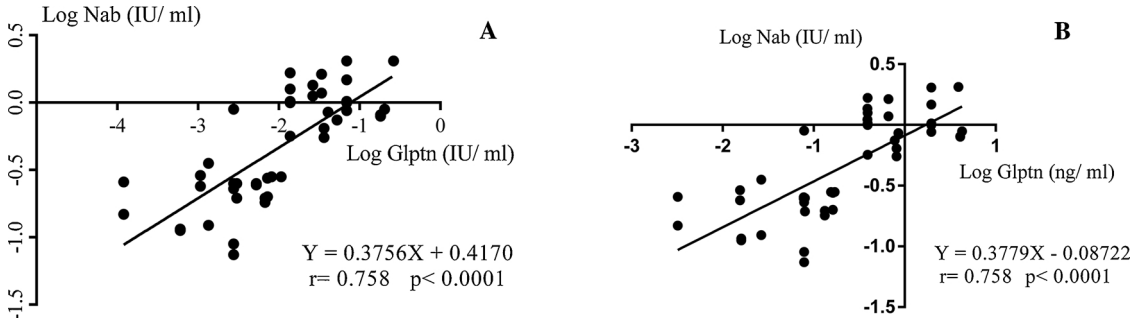

Fig. 1. (A) Linear regression of the Nab titers logarithms of mice (Log Nab) against the Glptn content (Log Glptn) of rabies vaccine batches against Refvac. (B) Linear regression of the Nab titers logarithms of mice versus the Glptn content (Log Glptn) of rabies vaccine batches against the Pasvac.

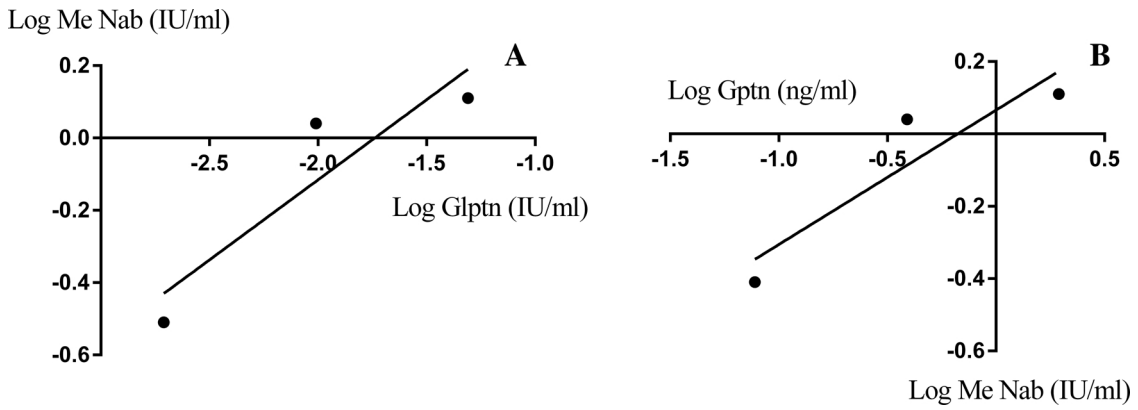

Fig. 2. (A) Linear regression of the Nab mean titers logarithms (Log Me Nab) against the Glptn content (Log Glptn) of rabies vaccine batches against Refvac. (B) Linear regression of the Nab titers logarithms (Log Me Nab) of mice immunized with BR014 and the Glptn content (Log Glptn) of rabies vaccine batches against Pasvac expressed in ng/ ml.

can be used to control the quality of inactivated human rabies vaccines in official control laboratories and by manufacturers. The SPT is rapid, efficient and in accordance with the immunogenicity induced in mice, and therefore may be recommended for validation as a potency test to replace the NIH test.

Conflict of interest

There is no conflict of interest of the authors of this paper.

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