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Development and pre-validation of a quantitative multi-dose serological assay for potency testing of inactivated rabies vaccines for human use

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ABSTRACT

It is mandatory to ensure the quality of biological products used in the prevention of rabies, a zoonosis with nearly 100% lethality. Fifteen million people receive post-exposure prophylaxis yearly. The vaccine batches are assessed by the National Institutes of Health (NIH) test which has several disadvantages such as significant variability and animal welfare issues. The estimation of immunogenicity based on titration of neutralizing antibodies (NA) is not applied to the human vaccine yet. Despite this, a satisfactory concentration of NA (0.5 IU/ml) can be used as a predictor of the clinical efficacy and for estimating rabies vaccine potency. The objective of this study was to develop and pre-validate a Serological Potency Test (SPT) using the modified Rapid Fluorescent Focus Inhibition Test (mRFFIT) to determine the potency of rabies vaccines for human use, demonstrating its relevance and reliability. The results show good agreement between the potencies determined by the SPT and the NIH test. The assay was able to distinguish between potent and sub-potent lots of vaccines. The results demonstrated that SPT is a viable candidate for validation and inclusion in pharmacopeias as a reduction and refinement for the NIH test.

1. Introduction

Rabies is an anthropozoonosis transmitted to humans by *Rabies lyssavirus*, Order *Mononegavirales*, family *Rhabdoviridae*. It can affect all mammals and is characterized by acute encephalitis that can lead to death in almost 100% of the cases (Rupprecht et al., 2002; Acha and Szyfres, 2003; Consales and Bolzan, 2007; ICTV, 2011). Although it was one of the first human diseases to have an experimentally developed vaccine used successfully in 1885 (Vodopija and Clarck, 1991), the World Health Organization (WHO) lists rabies among the top ten causes of human deaths in the world (WHO, 2014).

Historically, biologicals such as vaccines have been predominantly produced by complex biological processes (bacterial fermentation, cell culture, and embryonated eggs), from which reproducibility is challenging and carried out by potency verification methods that are as old as the first vaccines (Ranhein et al., 2015).

Rabies vaccines for human use must have a minimum potency of 2.5 IU/dose and the viral strain must demonstrate adequate immunogenicity (potency) for humans. For vaccine batch release purpose, batches are assessed by viral identification, sterility and viral titers (Brasil, 2010a; WHO, 2007; Council of Europe, 2015).

The potency assay evaluates the immunogenicity of rabies vaccines versus a reference vaccine by the NIH method (Brasil, 2010a; Wilbur and Aubert, 1996; Council of Europe, 2015). Briefly, this *in vivo* method consists of two intraperitoneal (ip) immunizations of mice groups seven days apart, followed by an intracerebral (ic) challenge with a standard virus (challenge virus standard - CVS). After the challenge, animals are monitored for 14 days and the relative potency of the test vaccine is calculated by comparing its 50% effective dose (ED50) with the ED50 of the reference vaccine (Wilbur and Aubert, 1996). Despite its capability to discriminate between potent and sub-potent lots, the NIH test has significant drawbacks such as inaccuracy, poor reproducibility, never been properly validated and severe ethical and animal welfare issues. To minimize or avoid the pain and suffering associated with the ic procedure, mice should be anesthetized (Hedrich and Bullock, 2004).

The WHO recommends that newly developed rabies vaccines must be submitted to clinical trials (WHO, 2007) and for the clinical evaluation of rabies vaccination and licensing, the vaccine must present a

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minimum potency of 2.5 IU per dose, inducing adequate neutralizing antibodies (NA) titers in humans which are evaluated by either the Rapid Fluorescent Focus Inhibition Test (RFFIT) or the Fluorescent Antibody Virus Neutralization Test (FAVN). An antibody titer of at least 0.5 IU/ml on days 14 and 28 or 30 after vaccination is generally considered appropriate. Therefore, the concentration of NA can be used as a clinical efficacy predictor of this product. Immunogenicity tests are already applied to veterinary vaccines, as an alternative to the challenge test, with immunization followed by titration of NA (Krämer et al., 2009, 2010; Council of Europe, 2013).

Validation of potency assays for vaccines is typically based on (1) the type of vaccine tested and (2) on specific details of analytical procedures, in which potency may mean antigen content or, more typically, biological activity. It consists of demonstrating the relevance and reliability of a method or process for a specific purpose (Organization for Economic Co-Operation and Development (OECD, 2005; International Conference on Harmonisation of Technical Requirements for Registration of pharmaceuticals for Human Use (ICH, 2005).

The validation process usually evaluates accuracy (or trueness), precision (repeatability and intermediate precision), specificity, detection and quantification limits, linearity, and measuring interval. The phases until test implementation are the pre-validation, which consists of an initial evaluation aiming at the adjustment, optimization, and standardization of the protocol and standard operational procedures. In this phase, preliminary relevance and reliability data are obtained to justify the formal validation study or validation itself (Organization for Economic Co-Operation and Development (OECD, 2005).

The pre-validation process compares the candidate test with the traditional assay to demonstrate reliability by determining assay precision, by means of inter-assay variability (intra- and inter-assay precision) and demonstrating the relevance. The results are compared to determine either sensitivity or agreement and the ability to differentiate potent from sub-potent batches. Subsequently, its transferability is assessed (Organization for Economic Co-Operation and Development (OECD, 2005).

Although some practices are used to reduce animal suffering such as the use of anesthetic agents, the NIH test, the only test accepted for releasing rabies vaccine lots for human use, is not in accordance with current animal welfare recommendations and the 3Rs strategy. In addition, the WHO recommends evaluating vaccines by the immunogenicity that is accepted for veterinary vaccines. Based on this, this research aims to develop and pre-validate a Serologic Potency Test (SPT) as a reduction and refinement of the NIH method.

2. Materials and methods

To develop and pre-validate the SPT, the same vaccine samples were used to perform the SPT and NIH test and results were compared. The SPT performance was submitted to statistical analysis to demonstrate its relevance and reliability.

2.1. Vaccine samples

Eight batches of human rabies vaccine (Rabvac - Sanofi Pasteur, França) approved in the NIH test were selected and labeled from A to H (Table 1). The freeze-dried Rabvac contains 0.5 ml diluent in one-dose vials, and is produced in Vero cells using the Wistar PM/WI38 1503-3 M strain of rabies virus, inactivated by betapropriolactone and purified. The minimum potency required is 2.5 IU/ dose. After reconstitution, five batches (A, B, D, E and H) were submitted to heat treatment 52° ± 1 °C for 48 h ± 10 min to obtain sub-potent vaccines (Table 2).

2.2. Reference vaccine

The Brazilian National Rabies Reference Vaccine BR014 (Refvac) is a commercial lot manufactured in October/2009 and it is the same product of the vaccine samples with the same strain of rabies virus. The BR014 was certified 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 an attributed titer of 9.0 IU per vial, according to the WHO recommendations (WHO, 2006).

Table 1
Rabies vaccine samples.

Vaccines	Potency (IU/ dose)	
	INCQS	Manufacturer
Vac A*	15.0	4.2
Vac B*	19.0	14.4
Vac C	8.0	4.8
Vac D*	nt	8.7
Vac E*	nt	6.8
Vac F	> 2.5	17.6
Vac G	nt	4.4
Vac H*	12.3	15.0

* Batches were submitted to heat treatment. nt– not tested.

2.3. Laboratory animals

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

For the SPT, mice were separated into three groups of five animals each for testing three dilutions of the vaccine samples. For the NIH test, 16 mice were used for each of the three vaccine dilutions and three groups of 10 mice were used for virus titration. The study was approved by the Fiocruz Institutional Animal Care and Use Committee (LW-17/16.).

2.4. NIH potency test (NIH)

Vaccine samples were tested by NIH as described in the Brazilian Pharmacopoeia (Brasil, 2010a). The assay consisted of two ip immunizations (0 and 7 day), with 0.5 ml of the dilutions (samples A–E and H: 1/25, 1/125 and 1/625; sample E assay 03/18: 1/250, 1/1250 and 1/6250; sample F assay 07/18: 1/1100, 1/5500 and 1/27500; sample G: 1/200, 1/1000 and 1/5000) of the samples or Refvac (1/50, 1/250 and 1/1250). On day 14, the challenge was performed by ic route with 32 50% lethal dose (LD50)/ 0.03 ml of challenge virus standard (CVS). The CVS was titrated simultaneously. The animals were observed for 14 days and upon development of rabies signs, humane endpoints were applied (Brasil, 2010a). The relative potencies of vaccine lots can be seen in Table 2.

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

The SPT protocol was adapted directly from the NIH for direct comparison. The ic challenge step was replaced by cardiac puncture bleeding under anesthesia and the number of animals was reduced from 16 to 5 per dilution. No viral titration was performed. For determining the NA titers against rabies, a total of 344 sera from SPT in 11 assay runs, vaccinated mice were evaluated by modified rapid fluorescent focus inhibition test adapted for 96-well microplate (mRFFIT) (Table S2). On day 14 after immunization, the animals were bled. After retraction of the clot, the blood samples were centrifuged at 200 g for 15 ± 1 min, the sera transferred to sterile cryovials, inactivated at 56 ± 1 °C for 30 ± 1 min, and kept at -20 ± 1 °C until being individually tested.

Table 2
Relative test and NIH against BR014.

Vaccine	SPT ap-WHO[1]				NIH			
	Regular		Heat treatment[2]		Regular		Heat treatment	
	Potency (UI/ dose)	Confidence intervals (%)	Potency (UI/ dose)	Confidence intervals (%)	Potency (UI/ dose)	Confidence intervals (%)	Potency (UI/ dose)	Confidence intervals (%)
A	3.61	19.2 – 500.9	0.00†	–	15.09	42.3–260.7	0.00†	–
B	6.39*	20.6 – 515.2	0.00†	–	19.44	43.0–248.2	0.00†	–
C	6.62	13.8 – 927.2	0.25	56.9 – 175.8	8.13	43.4–240.9	0.02	0.8–618.3
D	6.39	20.6 – 515.2	0.00†	–	8.70ƛ	46.0–229.7	nt	–
E	9.46	21.2 – 535.1	0.00†	–	6.80ƛ	50.0–198.5	0.05	2.2–427.5

Potency determination approach [1]cut-off ≥0.5 IU/ ml as the minimum seroconversion titre. Acceptance criteria: significant slope in the dose response curve; there are no significant deviations from linearity and parallelism; the ED50 of the reference and test vaccines lies between the lowest and the highest dose applied to the animals; the ED50 obtained for the reference vaccine remains within the 99% confidence limits in the control chart, the potency ≥2.5 IU/ dose and the lower confidence limit of the estimated potency is greater than 0.62 IU/ dose (25% of 2.5 IU/ dose).
*Sample with non-conform potency was repeated. ƛ Potency calculated by the manufacturer. [2]Heat treatment 52° ± 1 °C for 48 h ± 10 min. †The confidence interval could not be determined. nt – not tested.

The serum samples were two-fold serially diluted from 1/4 to 1/32 and the working virus suspension was diluted to contain 200 50% focus-forming doses. The plates were incubated at 36.5 ± 0.5 °C in a humidified incubator with 5% CO_2 for 90 min. Subsequently, BHK-21 at a concentration of 3.5×10^5 cells/ml was added and the plates were incubated for 22 h. The plates were washed with PBS, fixed with 80% acetone and incubated with the commercial 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 well (Brasil, 2010b; Moura et al., 2008). The NA titer was calculated using the probit analysis in CombiStats 5.0 (EDQM) and expressed in IU/ml.

In order to choose the most suitable calculation method, the relative potencies were determined in SPT with three calculation approaches. The WHO approach (ap-WHO), with a cut-off of ≥0.5 IU/ml as indicative of seroconversion titer (WHO, 2005); a model of parallel lines with the absolute NA titre in IU/ ml of each mouse (ap-TNA) was used. In the third, called the Advisory Committee on Immunization Practices (ACIP) approach, the cut-off ≥0.12 IU/ ml (Manning et al., 2008), was adopted as indicative of seroconversion.

The minimum potency requirement adopted for the SPT was ≥2.5 UI/dose, as in the NIH.

2.6. Statistical analysis

The Microsoft® Excel, GraphPrism®, MediCalc®, and CombiStats® were used for the statistical analysis. The potency results were logarithmic transformed to improve the normal distribution fitting when necessary.

The weighted least squares method and probit analysis were used for transforming the sigmoid dose-response curve into a straight line that can be further analyzed by regression through either least squares or maximum likelihood (Finney, 1971).

The normal distribution of the results was evaluated by the Omnibus D'Agostino & Pearson normality test (Miot, 2017). Similarity of the corresponding potencies between the SPT and NIH were estimated using: student *t*-test for paired samples, analysis of variance (ANOVA), simple linear regression, correlation, Cohen Kappa coefficient, Lin correlation coefficient (ρc), and confidence intervals (CI).

In the SPT relevance study, student *t*-test, ANOVA, Pearson correlation, sensitivity, specificity, accuracy, positive and negative (PPV and NPV) predictive values were applied for describing the SPT performance while the Cohen Kappa agreement index evaluated the concordance of the results with NIH and mainly its ability to differentiate potent from sub-potent batches. The Cochran *C* test was performed to determine homogeneity of variances.

The reliability was determined by calculating the intra- and inter-assay variances from the logarithmic transformed NA titers of the Refvac by dilution in three runs of mRFFIT. Intra- and inter-assay geometric coefficients of variation (gCV%) were obtained. The SPT precision was also demonstrated by comparing it with the potencies obtained in NIH by the ρc and 95% CI.

3. Results

3.1. Standardization of the serological potency test

The vaccine samples were submitted to the SPT and the sera of immunized mice were titrated to determine the vaccine relative potency and to demonstrate SPT relevance and reliability. The ap-WHO presented the most suitable calculation for relative potencies and it was chosen (Supplemental Table S1).

3.1.1. Antibody titration by mRFFIT

Supplemental Table S2 shows the raw data for the *in vitro* rabies NA titers of 344 serum samples from vaccinated mice. Aiming at pre-validating the SPT, 11 test runs were performed and the data were used to calculate the potency by the SPT. The SPT results for the samples rendered sub-potent by heat treatment were in agreement with the NIH test. The NIH test was not performed with the sub-potent D vaccine (Table 2).

3.2. Pre-validation of the serological potency test

3.2.1. Relevance

The ap-WHO was used in material and methods by statistical analyses. The resulting of NIH and SPT were compared by one-way ANOVA to determine whether there was a significant difference between them and there was no statistically significant differences between NIH and SPT (F 3.067; ρ 0.1125) serological potency, although a trivial heterogeneity was observed between the absolute values of the vaccine relative potencies (F 4.096; 0.0079). The homogeneity of variances between SPT and NIH tests for vaccines A, B and C was considered homogeneous (*C* 0.7256).

A relevance study was performed with the potencies shown in Table 2. In this evaluation, all the results were concordant when the relative potency was calculated in SPT. The relative potencies were not significantly different as shown by the paired student *t*-test at 5%. The comparison between SPT and NIH showed agreement between the results, including for sub-potent vaccines. The difference between the means was -0.1094, with a standard deviation of 0.2308 (*t* 1.423, *p* 0.2019, *r* 0.9267).

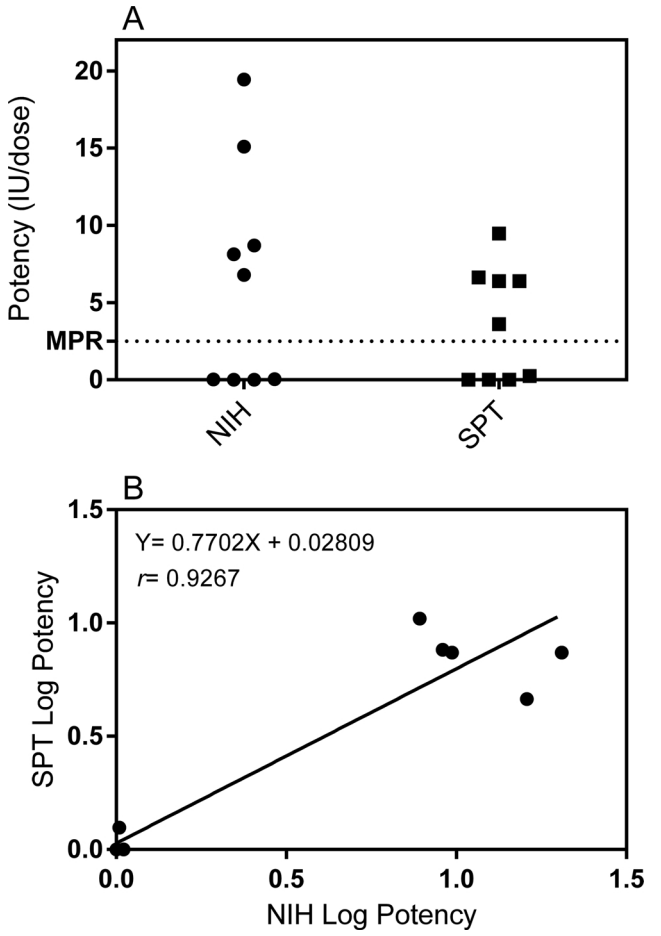

Fig. 1. (A) The relative potency calculation approaches; MPR - minimum potency requirement (≥2.5 IU/ dose). Pearson correlation of the potency logarithms (B) between SPT ap-WHO and NIH test.

Fig. 1 shows the relative potency by NIH and SPT of the eight batches of Rabvac (samples A–H) plus the Refvac, totaling nine samples (Fig. 1A). The potency determination by SPT showed that five batches were compliant (≥2.5 IU/dose). The Pearson coefficient was significant ($p < 0.05$) while SPT and NIH were highly correlated with r 0.9267 (r^2 0.8589) and linear regression equation Y = 0.7702X + 0.02809 (Fig. 1B).

After categorizing concordant (pass) and discordant (fail to pass) samples, the SPT and NIH results were compared for determining sensitivity, specificity, accuracy as well as positive (PPV) and negative (NPV) predictive values for profiling SPT performance. In this comparison all indexes found were 100%, demonstrating a perfect agreement between SPT and NIH test results. The Cohen Kappa indexes for the SPT results were also verified using the categorized data and the relationship between SPT and NIH presented a great concordance strength (Kappa 1.0). The SPT precision was demonstrated by the ρc considered satisfactory (0.88) while Pearson coefficient (ρ) was excellent (0.93). The SPT had better performance, displaying 93% accuracy under ideal conditions, by the Lin Concordance Coefficient, thus agreeing with the NIH when identifying sub-potent batches. Also, trueness (capacity to identify the true value) was evaluated as excellent (0.95) (Table 3).

The ρc graph of the SPT and NIH are shown in Fig. 1B. A satisfactory agreement is observed, demonstrating a higher concordance and correlation. The CI of vaccine potencies for the NIH and SPT are plotted in Fig. 2. To demonstrate the calculation precision, the lower and upper confidence (25% and 400%) intervals for the calculated potencies are plotted as well, as recommended for the NIH test (Brasil, 2010a). The

Table 3
Summary of statistical analysis of the serological potency test against the NIH test.

Descriptors	Values	Confidence intervals
Sensitivity	100.00	47.82–100.00
Specificity	100.00	39.76–100.00
Positive Predictive Value	100.00	–
Negative Predictive Value	100.00	–
Accuracy	100.00	66.37–100.00
Kappa[a]	1.0000	–
Concordance (%)	100.00	–
pc[b]	0.88	0.63–0.96
Precision (Pearson ρ)	0.93	–
Trueness	0.95	–

[a] Cohen's Kappa Coefficient.
[b] Lin's Concordance Coefficient. Source on line available in: https://www.graphpad.com/quickcalcs/kappa2/ and https://www.medcalc.org/calc/diagnostic_test.php.

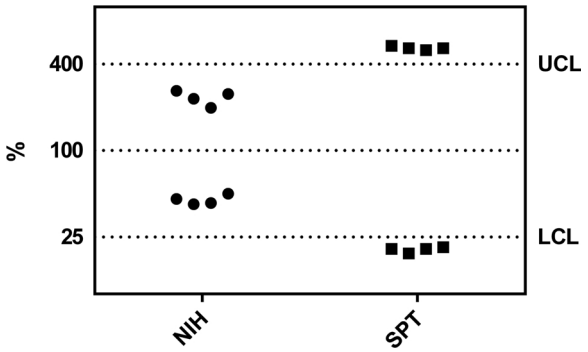

Fig. 2. 95% Confidence intervals upper (UCL) and low (LCL) control limits of the relative potencies determined in the NIH and Serologic Potency Test. The sample C, which was excluded due to high variability, is not represented in these figures. n = 4.

vaccine C interval was very wide, therefore, this outlier sample was excluded due to high variability, allowing to elaborate a more didactic chart. It was observed that the potency values calculated in SPT were outside the CI.

3.2.2. Reliability

Pools of sera from three different dilutions (1:50, 1:250 and 1:1250) of the Refvac from two independent SPT assay runs (01 and 02/17) were tested in mRFFIT in three new assay runs (SPT 06, 08 and 09/18). The results were used to evaluate the reliability by determining the intra- and inter-assay precisions. Intra- and inter-assay variances of the NA titer logarithms were calculated by dilution. 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

The present study was carried out to pre-validate a serological potency test (SPT) for human rabies vaccines, in consonance with other researchers (Krämer et al., 2013). The SPT uses fewer animals (n = 15) compared to the NIH test (n = 48), providing a considerable reduction (69%) of the use of animals. In addition, a refinement is achieved by replacing the ic challenge by blood sample collection under anesthesia and reducing the test duration by at least two weeks.

Three different potency calculation approaches were used to compare the NIH and SPT results. The calculation using the ap-WHO produced better agreement between SPT and NIH, when the interpretation was based on an indirect quantal endpoint with all or nothing response (seroconversion or no seroconversion). This was based on the WHO

recommendation of 0.5 IU/ ml rabies neutralizing antibodies titer as the clinical predictor of vaccination response (WHO, 2007). In all runs in which SPT ap-WHO was used, valid assays were obtained and this approach was chosen for the pre-validation study.

The SPT relevance had great sensitivity, specificity, accuracy, PPV and NPV, indicating a perfect performance. The correlation coefficient (r 0.9267) showed high agreement with NIH as observed by other researchers for *in vitro* assay (ELISA) (Gamoh et al., 1996; Perrin et al., 1990; Gibert et al., 2013; Morgeaux et al., 2017). The SPT also had a higher correlation compared to the fluoroimmunoassay test (Lin et al., 2017) and other serological tests (Krämer et al., 2009; Winsnes et al., 2003; Hendriksen et al., 1991). A few differences were observed when comparing the relative potencies obtained by SPT and NIH. However, full compliance of absolute potency values cannot be expected due to the high lack of accuracy inherent to animal and cell assays (Krämer et al., 2013; Stokes et al., 2012; Schrock, 2012; Rieder et al., 2010). Despite this, the SPT and NIH results were highly correlated as required by Stokes et al. (2012).

A modern approach for comparing assays is the understanding that the substitute method should not be required to have a significant correlation with the reference method, since the NIH has inherent variability. The new test should agree with the clinical potency of the vaccine and should be able to discriminate between potent and sub-potent lots. This is why some authors refer to “agreement” rather than “correlation” (European Partnership for Alternative Approaches (EPAA, 2012), although many studies still use correlation as a method of analysis (Krämer et al., 2009, 2013; Morgeaux et al., 2017). As the NA titer can be used as a clinical efficacy predictor of rabies vaccines in humans (WHO, 2005), the SPT can directly predict the vaccine clinical potency, a requirement referred by the EPAA (2012).

The SPT sensitivity was higher than that observed by Chabaud-Riou et al. (2017) in serological assays using ELISA, and the 94% reported by Sigoillot-Claude et al. (2015). This high sensitivity revealed one of the major SPT advantages regarding recognizing rabies antigen as identified by other researchers (Sigoillot-Claude et al., 2015). Another important factor already mentioned by others is the different nature of endpoint used in each test: NA titers in SPT versus survival rates in NIH (Krämer et al., 2013). In this matter, although it clearly influenced SPT, we add that the method uses probit analyses for potency determination, as NIH, minimizing this effect.

The SPT results of untreated vaccines were examined in conjunction with the sub-potent samples. The use of sub-potent products or borderline samples is important to assess the methodology (Milne and Buchheit, 2012). Future validation studies should include borderline samples classified by NIH to access the SPT sensitivity. The SPT and NIH results were concordant, i.e., the method was able to distinguish between potent and sub-potent batches, and in agreement with NIH as previously described (Krämer et al., 2013; Gibert et al., 2013) but in disagreement with previous studies (Servat et al., 2015) in which a serological potency assay using the mRFFIT and FAVN tests failed to identify regular and sub-potent lots. Other groups found only satisfactory agreement for identifying nonconforming lots (Morgeaux et al., 2017; Chabaud-Riou et al., 2017). Some researchers have found sensitivity indexes varying from 55 to 68% (Servat et al., 2015), and from 47 to 90% (Krämer et al., 2010), both lower than the value observed in this study.

Differences were observed between the individual responses of the mice in the test vaccine and VacRef groups, where there was a better response in seroconversion. In particular, significant differences may indicate a change in the immune status of the mice or in other aspects of animal husbandry (van der Ark et al., 2000). High, low, and non-responder animals were observed in the present study. Non-responders were found primarily in animal groups vaccinated with lower vaccine doses as previously observed (Krämer et al., 2013). The exclusion of non-responders may influence the test result, since this failure may be not entirely intrinsic to the animal, but may be due to a sub-potent vaccine (Krämer et al., 2010).

There is a gap in the detection of true non-responders, raising questions about a given vaccine batch having low potency or the animal being non-responder. Cellular kinetics studies are necessary to complement and identify true non-responders as suggested by some researches (Bordignon et al., 2002), thus determining with confidence how many individuals can be discarded from the potency calculations.

The SPT relevance could be demonstrated by the perfect performance (The United States Pharmacopoeia (USP, 2017). The Cohen Kappa coefficient indicated great concordance strength of SPT when adopting the concordance tendency rather than the correlation (Stokes et al., 2012; Schiffelers et al., 2014).

The SPT accuracy of 100% obtained from sensitivity calculation was higher than previously found, with CI comparable to that of ELISA (Chabaud-Riou et al., 2017). In the present study, to compare SPT and NIH directly, SPT was performed as a direct adaptation of the NIH protocol, including dose range, as used in INCQS, but only 5 mice per dilution were used in 3 dilutions per assay. The CIs were broader than in NIH, differing from those found by other authors (Krämer et al., 2013). This result shows the need for adjusting the dose (dilutions) range to be used in the SPT, in order to improve the similarity of responses between reference and test vaccines, a basic assumption for biological assays. Once the proof of concept was established, the method can be transferred to at least one additional laboratory with the adoption of a fourth dose which could resolve this adjustment.

This wider CIs may be also probably explained by the variation within the group of animals in SPT. Some mice developed titers higher than others in the same group and in the Refvac group, which is in accordance to previous studies (Krämer et al., 2009), NIH is also influenced by the variability of individual immune responses although not as obvious as in the SPT. Another determinant factor for the wider CIs found was that the reference standard used in the SPT proposed here was calibrated in the NIH. Therefore, prior calibration of the standards in the SPT itself is strongly recommended for a future validation study.

The sample failure to reach minimum potency requirements was readily recognized, demonstrating SPT relevance. This aspect is of particular importance with regard to the release of anti-rabies vaccine batches (Krämer et al., 2009).

Reliability is a pre-requisite for the validity of serological assays due to its ability to estimate the vaccine protective activity, which was readily obtained with the proposed SPT (Hendriksen, 1995). The assay precision assessed by the inter-assay geometric coefficients of variation was about 30%, indicating the good performance of SPT, and considered adequate (Kostense et al., 2012). Researchers have previously reported intra-assay precision varying from 6.1 to 35.5% and inter-assay from 8.6 to 37.6% per laboratory for a Pertussis Serological Potency test (van der Ark et al., 2000); similar values were found for the SPT, 31.99 -31.65% and 31.82% for intra- and inter-assay variation, respectively.

In conclusion, the SPT is an accurate, specific and sensitive model for estimating potency and confirming compliance with specifications of rabies vaccines for human use and it is a candidate test for future research and validation. The assay was able to distinguish between potent and sub-potent vaccine lots. There was satisfactory agreement between the corresponding potency in the NIH confirming preliminary relevance and reliability data. It can also reduce test duration, streamlining batch release and improving animal welfare, while ensuring the efficacy of human vaccines.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.10.003.

References

Acha, P.N., Szyfres, B., 2003. Rabia. In: Acha, P.N., Szyfres, B. (Eds.), Zoonosis y enfermedades transmissibles comunes al hombre y a a los animales, 3rd ed. Organización Panamericana de la Salud. Oficina Sanitaria Panamericana, Oficina Regional de la Organización Mundial de la Salud, Washington, pp. 351–383.

Bordignon, J., Comin, F., Ferreira, S.C.P., Caporale, G.M.M., 2002. Lima Filho JHC, Zanetti CR. Calculating rabies virus neutralizing antibodies titres by flow cytometry. Rev. Inst. Med. Trop. S Paulo 44 (3), 151–154.

Brasil, 2010a. 5th ed. Farmacopeia Brasileira. Vacina Raiva Inativada, vol. 2. Agência Nacional de Vigilância Sanitária, Brasília, pp. 1366–1368.

Brasil, 2010b. 5th ed. Farmacopeia Brasileira. Soro antirrábico. Farmacopeia Brasileira, vol. 2. Agência Nacional de Vigilância Sanitária, Brasília, pp. 1297–1298.

Chabaud-Riou, M., Moreno, N., Guinchard, F., Nicolai, M.C., Niogret-Siohan, E., Seve, N., et al., 2017. G-protein based ELISA as a potency test for rabies vaccines. Biologicals 46, 124–129.

Consales, C.A., Bolzan, V.L., 2007. Rabies review: immunopathology, clinical aspects and treatment. J. Venom. Anim. Toxins Incl. Trop. Dis. 13 (1), 5–38.

Council of Europe, 2013. The European Pharmacopoeia. Rabies Vaccine (inactivated) for Veterinary Use, 8.0 ed. Council of Europe. European Department for the Quality of Medicines within the Council of Europe, Strasbourg.

Council of Europe, 2015. The European Pharmacopoeia. Rabies Vaccine for Human Use Prepared in Cell Cultures, 8.2 ed. Council of Europe. European Directorate for the Quality of Medicines and Healthcare, Strasbourg, pp. 3952–3954.

European Partnership for Alternative Approaches (EPAA), 2012. Application of the 3rs and the Consistency Approach for Improved Vaccine Quality Control. [Acessed in 2014 Sep. 12]. Available from:. The Vaccines Consistency Approach Workshop, Arcachon, France . Acesso em: 12 set. 2014. https://circabc.europa.eu/sd/a/6cfc6e14-4c81-4981-a424-5b51b982da1d/flash-report-vaccines-workshop-october-2012_en.pdf.

Finney, D.J., 1971. Probit Analysis, 3rd ed. Cambridge University Press, New York, Ny 10022, 32 E. 57th St.

Gamoh, K., Senda, M., Itoh, O., Muramatsu, M., Hirayama, N., Koike, R., et al., 1996. Use of ELISA for in vitro potency test of rabies vaccines for animal use. Biologicals 24, 95–101.

Gibert, R., Alberti, M., Poirier, B., Jallet, C., Tordo, N., Morgeaux, S., 2013. A relevant in vitro ELISA test in alternative to the in vivo NIH test forhuman rabies vaccine batch release. Vaccine 31, 6022–6029.

Hedrich, H.J., Bullock, G., 2004. The Laboratory Mouse. Elsevier Academic Press, London.

Hendriksen, C.F.M., 1995. Development, validation and acceptance of alternative methods in the quality control of vaccines: a case report. Toxicol. In Vitro 9 (6), 815–819.

Hendriksen, C.F.M., van der Gun, J.W., Marsman, F.R., Kreeftenberg, J.G., 1991. The use of the in vitro toxin binding inhibition (ToBI) test for the estimation of the potency of tetanus toxoid. Biologicals 19, 23–29.

ICTV 9th Report, 2011. International Committee on Taxonomy of Viruses - ICTV. [homepage in internet] [Accessed in 2017 Sep. 01]. Available from:. . https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/201/rhabdoviridae.

International Conference on Harmonisation of Technical Requirements for Registration of pharmaceuticals for Human Use (ICH), 2005. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1). pp. 17.

Kostense, S., Moore, S., Companjen, A., Bakker, A.B.H., Marissen, W.E., von Eyben, R., et al., 2012. Validation of the rapid fluorescent focus inhibition test for rabies virus-neutralizing antibodies in clinical samples. Antimicrob. Agents Chemother. 56 (7), 3524–3530.

Krämer, B., Schildger, H., Behrensdorf-Nicol, H.A., Hanschmann, K.M., Duchow, K., 2009. The rapid fluorescent focus inhibition test is a suitable method for batch potency testing of inactivated rabies vaccines. Biologicals 37, 119–126.

Krämer, B., Bruckner, L., Daas, A., Milne, C., 2010. Collaborative study for validation of a serological potency assay for rabies vaccine (inactivated) for veterinary use. Pharmeur. Biol. Sci. Notes 2, 37–55.

Krämer, B., Kamphuis, E., Hanschmann, K.-M., Milne, C., Daas, A., Duchow, K., 2013. A multi-dose serological assay suitable to quantify the potency of inactivated rabies vaccines for veterinary use. Biol 41, 400–406.

Lin, G., Chen, S., Zhao, H., Liang, J., Deng, Q., Liang, R., et al., 2017. A time-resolved fluoroimmunoassay to assay the rabies virus glycoprotein: application for estimation of human rabies vaccine potency. Sci. Rep. 7 (7288), 1–9. https://doi.org/10.1038/s41598-017-07687-7.

Manning, S.E., Rupprecht, C.E., Fishbein, D., Hanlon, C.A., Lumlertdacha, B., Guerra, M., et al., 2008. Human rabies prevention-United States, 2008: recommendations of the Advisory Committee on immunization practices. MMWR Recomm. Rep. 57 (RR-3), 1–28.

Milne, C., Buchheit, K.H., 2012. EDQM's 3R activities in the field of quality control of vaccines. ALTEX Proc. 1/12, 65–69.

Miot, H.A., 2017. Assessing normality of data in clinical and experimental trials. J Vasc. Bras. 16 (April-June 2), 88–91. https://doi.org/10.1590/1677-5449.041117.

Morgeaux, S., Poirier, B., Ragan, C.I., Wilkinson, D., Arabin, U., Guinet-Morlot, F., et al., 2017. Replacement of in vivo human rabies vaccine potency testing by in vitro glycoprotein quantification using ELISA – results of an international collaborative study. Vaccine 35, 966–971.

Moura, W.C., Gallina, N.M., Fuches, R.M., Romijn, P.C., Leite, J.P., 2008. Validation of a virus neutralization potency test in BHK-21 cells for rabies immunoglobulins in a two-center study. J. Virol. Methods 54, 7–13.

Organization for Economic Co-Operation and Development (OECD), 2005. Nº 34. Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment. Paris. .

Perrin, P., Morgeaux, S., Sureau, P., 1990. In vitro rabies vaccine potency appraisal by ELISA: advantages of the immunocapture method with a neutralizing anti-glycoprotein monoclonal antibody. Biologicals 18, 321–330.

Ranhein, T., Mozier, N., Egan, W., 2015. Vaccine potency assays. In: Nunnally, B.K., Turula, V.E., Sitrin, R.D. (Eds.), Vaccine Analysis: Strategies, Principles and Control. Springer-Verlac, Berlin, pp. 521–542.

Rieder, N., Gazzano-Santoro, H., Schenerman, M., Strause, R., Fuchs, C., Mire-Sluis, A., et al., 2010. The roles of bioactivity assays in lot release and stability testing. Bioprocess. Int. 8 (6), 33–42.

Rupprecht, C.E., Hanlon, C.A., Hemachudha, T., 2002. Rabies re-examined. Lancet Infect. Dis. 2, 327–343.

Schiffelers, M.J., Blaauboer, B., Bakker, W., Hendriksen, C., 2014. Replacing the NIH test for rabies vaccine potency testing: a synopsis of drivers and barriers. Biologicals 42, 205–217. https://doi.org/10.1016/j.biologicals.2014.04.001.

Schrock, R.D., 2012. Cell-ased potency assays: expectations and realities. BioProcess. J. 11 (3), 4–12. https://doi.org/10.12665/J113.

Servat, A., Kempff, S., Brogat, V., Litaize, E., Schereffer, J.-L., Cliquet, F., 2015. A step forward in the quality control testing of inactivated rabies vaccines - extensive evaluation of european vaccines by using alternative methods to the in vivo potency tests. ATLA 43, 19–27.

Sigoillot-Claude, C., Battaglio, M., Fiorucci, M., Gillet, D., Vimort, A.S., Giraud, Y., et al., 2015. A versatile in vitro ELISA test for quantification and quality testing of infectious, inactivated and formulated rabies virus used in veterinary monovalent or combination vaccine. Vaccine 33 (32), 3843–3849. https://doi.org/10.1016/j.vaccine.2015.06.091.

Stokes, W., McFarland, R., Kulpa-Eddy, J., Gatewood, D., Levis, R., Halder, M., et al., 2012. 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 40, 369–381.

The United States Pharmacopoeia (USP). Development and Design of Biological Assays < 1032 > . USP 35 – NF 30:162, 2017. [Accessed in 2017 May 15]. Available from: http://www.usp.org/sites/default/fies/usp_pdf/EN/2010-03-25_1032_PF36(4)_w_line_numbers.pdf.

van der Ark, A., van Straaten-van de Kappelle, I., Ólander, R.M., Enssle, K., Jadhav, S., van der Donk, H., Hendriksen, C., 2000. The Pertussis Serological Potency test. Collaborative study to evaluate replacement of the mouse protection test. Biologicals 28 (2), 105–118.

Vodopija, I., Clarck, H.F., 1991. Human vaccination against rabies. In: Baer, G.M. (Ed.), The Natural History of Rabies, 2nd ed. CRC press, pp. 571–595.

Wilbur, L.A., Aubert, M.F.A., 1996. The NIH test for potency. In: Meslin, F.-X., Kaplan, M.M., Koprowski, H. (Eds.), Laboratory Techniques in Rabies, 4th edition. WHO, Geneva, pp. 360–368.

Winsnes R, Sesardic D, Daas A, Behr-Gross M-E. Collaborative Study for the Validation of Serological Methods for Potency Testing of Diphtheria Toxoid Vaccines Part 1. Pharmeuropa Bio 2003–2; 35–68.

World Health Organisation, 2005. WHO Expert Consultation on Rabies, First Report. Geneva, Switzerland; Report No.: TRS 931.

World Health Organisation, 2006. Recommendations for the Preparation, Characterization and Establishment of International and Other Biological Reference Standards. WHO Technical Report Series, Geneva No. 932.

World Health Organization, 2007. Recommendations for Inactivated Rabies Vaccine for Human Use Produced in Cell Substrates and Embryonated Eggs. no. 941 [Accessed in 2018 Jun 19]. Available from:. WHO Technical Report Series, Geneva, pp. 109–110. http://www.who.int/biologicals/publications/trs/areas/vaccines/rabies/Annex%202%20inactivated%20rabies%20vaccine.pdf?ua=1.