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Research Article

A Semi-quantitative Serological Method to Assess the Potency of Inactivated Rabies Vaccine for Veterinary Use*

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Potency is one of the most important indexes of inactivated vaccines. A number of methods have been established to assay the potency, of which the NIH test and single-dose mouse protection test are the "prescribed methods". Here, we report a method to semi-quantitatively assay the potency of an inactivated rabies vaccine, which uses fewer animals and takes less time to complete. Depending on the quality requirements of a vaccine (e.g. minimum potency), a rabies reference vaccine is, for example, diluted to the minimum potency, and 50 μL of the dilution is taken to inoculate 10 mice. The same amount of the test rabies vaccine is inoculated into another 10 mice. After two weeks, all mice are bled and serum samples are assayed for viral neutralizing antibody by the fluorescent antibody virus neutralization (FAVN) test. By comparing the median and interquartile range of antibody titers of the reference vaccine with those of the test vaccine, the test vaccine potency can be semi-quantitatively judged as to whether it is in accord with the required quality. The reliability of this method was also confirmed in dogs. The procedure can be recommended for batch potency testing during inactivated rabies vaccine production.

Rabies; Inactivated vaccine; Potency assay; Semi-quantitative method

Rabies is a generally fatal and incurable zoonotic disease caused by rabies virus and is usually transmitted to humans through the bite of a rabid dog[26]. Human and animal vaccination remains the most successful public health intervention. Since the emergence of inactivated rabies brain-tissue vaccines (*Fermi*, *Hempt* and *Semple* types) in the early 20th century, inactivated vaccines have been the most commonly used anti-rabies biologics for humans and domestic animals[25]. The potency of an inactivated rabies vaccine, primarily determined by the amount and concentration of virus antigen, is an indispensable evaluation index in the production process[13]. A number of methods, such as the NIH test or single-dose mouse protection test (a modified protocol of the NIH method)[18,24], Rabies peripheral challenge (RPC) test[27], Habel test[12] and single radial immunodiffusion test[5], have been established to assay the potency. Of these, the NIH test and the single-dose mouse protection test recommended by the World Health Organization (WHO) and the European Pharmacopoeia (EP) respectively are the most commonly used methods for quality control of inactivated rabies vaccines[18, 24].

However, in the NIH test, single-dose mouse protection test or RPC test, hundreds of mice are required for challenge with rabies virus strain CVS two or four weeks after immunization, and results are acquired over a whole test period of 30-45 days. In addition, poor precision, significant variability and ethical animal welfare problems in both tests have been widely reported[1,2,23]. Therefore, in order to establish a suitable alternative method to reduce animal distress and number, bear less experimental uncertainties, and be faster and easier to perform, some *in vitro* techniques have been developed such as ELISA and serological potency assay methods[4, 8, 9, 11, 14-17, 19-22].

Enzyme-linked immunosorbent assays (ELISAs) based on rabies-related polyclonal or monoclonal antibodies are designed to assess rabies virus glycoprotein content (the only protective antigen) for determination of the potency of inactivated vaccines, but they reflect only the reactivity of antigens *in vitro* and are inadequate to evaluate the immunogenicity *in vivo*.

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For improving vaccine production efficiency, it is important to have a way to rapidly determine whether the potency of a batch of vaccine satisfies the requirements, e.g., ⩾1 IU/dose, or ⩾2 IU/dose or ⩾3 IU/dose, whereas it is not absolutely necessary to know the exact value of the potency of a product.

Serological potency assays can compensate for the deficiency of ELISAs by quantifying the titers of rabies-neutralizing antibody in serum samples in the vaccinated animals. A modified rapid fluorescent focus inhibition test (mRFFIT) has been used for potency assaying of inactivated rabies vaccines[14, 15], and a good correlation between the results of the challenge test and the mean titers determined by the mRFFIT has been obtained. In the mRFFIT, the potency of test vaccine was determined by comparing mean values of serum antibody titers of the test and reference vaccines. The mean value is generally used when data (i.e., serum antibody titers) follows a normal distribution. However, the data cannot always be analyzed by a parametric method of statistics in practice, especially when the variations of antibody titer among individuals is high, which actually happens. So the method can not be reliably performed in practical vaccine production.

Here we report a method based on the median or interquartile range of antibody titers determined by fluorescent antibody virus neutralization (FAVN) test to semi-quantitatively assay the potency of inactivated rabies vaccines through intramuscular immunization of mice, using fewer animals and a shorter time than the standard methods.

MATERIALS AND METHODS

Reference and test vaccines

The 4th batch of the Biological Reference Preparation (BRP) with an assigned titer of 11 international units per vial (IU/vial) was provided by the European Directorate for the Quality of Medicines (EDQM, Strasbourg, France). Reference vaccines were stored at -20 ºC and reconstituted with 1 mL deionized water for injection. All reference dilutions indicated in the text were prepared with phosphate buffered saline (PBS) from these stock solutions (Table 1).

Table 1. Vaccines for mice immunization

Groups	Vaccines (1mL/dose)	Potency requirement (IU/mL)
Vac 1	Test vaccine 1	⩾2.0
Vac 2	Test vaccine 2	⩾1.0
Vac 3	Test vaccine 3	⩾2.0
Vac 4	Test vaccine 4	⩾2.0
Reference vaccine*	Biological Reference Preparation (BRP), the European Directorate for the Quality of Medicines (EDQM)	11 IU/vial

* Reference vaccine was diluted into reference 1 (1 IU/mL), reference 2 (2 IU/mL) and Reference 3 (3 IU/mL). To avoid potential conflicts of interest, the four test vaccines were respectively named as test vaccine 1–4.

As shown in Table 1, test vaccines were inactivated rabies vaccines for veterinary use from 4 different manufacturers, including Nobivac® Rabies (Batch No. A154A01, Intervet International B.V), Rabvac™ 3 (Batch No. 1215337A, Fort Dodge Animal Health), Rabisin-R (Batch No. L369409, Merial SAS. France) and Rabigen® mono (Batch No. 33XZ, Virbac Laboratories).

Challenge virus and standard serum

Rabies challenge virus standard CVS-24, a fixed laboratory strain, was propagated in mouse brains, and vials containing CVS suspensions were stored at -80 ºC. The intracerebral (I.C.) LD_{50}/0.03mL of the virus seed was $10^{4.50}$ in mice. Dilutions of CVS-24 were made in distilled water supplemented with 2% horse serum. CVS-11 virus was grown in BHK-21 cells and used in the neutralization test to monitor the neutralizing antibody titer. The $TCID_{50}$/mL of the virus seed was $10^{7.25}$ in BHK-21 cells.

The standard anti-rabies serum was obtained from Agence Française de Sécurité Sanitaire des Aliments (AFSSA, Nancy, France). A negative control serum was prepared from unvaccinated mice.

Mouse potency test

The test was performed according to the standard operating procedure (SOP) of the EP[18]. In brief, SPF out-bred mice, strain Kunming, at the age of 4 weeks, were selected at random and placed into groups of 10 animals. Four five-fold dilutions of reference and test vaccines were freshly prepared. On day 0, each group was injected intraperitoneally with 0.5 mL of a vaccine dilution. At the same time, the challenge dose (mouse intracerebral LD_{50}) of CVS-24 suspension was measured in control mice and calculated using the Spearman-Kärber formula. On the 14th day after vaccination, each treated mouse was injected intracerebrally with 0.03 mL of a CVS-24 suspension containing approximately 50 times of mouse LD_{50}. Mice with clear rabies symptoms were euthanized to avoid unnecessary suffering. The number of mice dying of rabies between the 5th and 14th day after challenge was recorded. The relative potency of the test vaccines was calculated by probit analysis using CombiStats software (EDQM).

Animals and immunization

Four-week-old Kunming mice were purchased from Changchun H&N Animal Breeding Center for Medicine (China) and divided in a randomized manner into seven groups (10 mice/group); i.e., Vac 1, Vac 2, Vac 3, Vac 4, Reference 1, Reference 2 and Reference 3 (Table 1) in the serological potency assay. According to prior experience[14,15,28], each mouse was injected with 0.05 mL of vaccine in the upper hind leg using a 29G * 1/2″ insulin syringe (BD Ultra-Fine™, batch 0116106, USA), and peripheral serum samples were collected from the retro-orbital plexus on the 14th day after treatment[6].

To demonstrate the vaccine potency assayed by the serological method in mice, unvaccinated beagles (3–4 months old) were divided into 7 groups (6 dogs /group); i.e. Vac 1–4 and reference 1–3 (Table 1). Each dog was inoculated with one dose of vaccine in the triceps brachii muscle of the right front leg, and serum samples were collected from the right front leg vein on the 14th day after vaccination.

All the mice and dogs used in this study were treated in accordance with DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes.

FAVN test for potency testing of vaccines

The antibody titers of serum samples were measured by the FAVN test performed according to the method described by Cliquet *et al.*[3]. Briefly, 3-fold serial dilutions of both the serum samples and the controls were prepared in microplates. Each serum dilution was tested in quadruplicate. 50 μL (100 $TCID_{50}$) of challenge rabies virus CVS-11 was also added to each well. After 1 h incubation at 37 ℃ in a 5% CO_2 humidified incubator, 50 μL of BHK-21 cell suspension containing 4×10^5 cells/mL was added to each well and the plates were incubated for 48 h at 37 ℃. After fixing the cells in 80% cold acetone for 30 min at room temperature, the plates were stained by adding FITC-conjugated anti-rabies monoclonal antibody (prepared in our laboratory), incubating at 37 ℃ for 30 min and then washing with PBS-Tween 20. The plates were examined by fluorescence microscopy (Olympus Corp., Tokyo, Japan). Presence or absence of fluorescent foci in the cells was recorded. Endpoints were calculated as the inverse of the highest dilution showing no fluorescence, using the Spearman-Kärber formula. A standard anti-rabies serum (AFSSA, Nancy, France) was included as a positive control in all assays, and data were calculated as IU/mL of rabies virus-neutralizing antibody.

Statistics

The median (Md) and interquartile range (IQR) of serum antibody titers of each group were calculated using the SPSS 16.0 program (SPSS Inc., Chicago, IL, USA) for Windows by a non-parametric method[10]. By comparing the Md of the reference vaccines with that of the test vaccine, the potency of test vaccine can be semi-quantitatively determined within a range; e.g. >2 IU/dose. However, when the Md of test vaccine is equal to that of the reference, the IQRs should be compared to determine the potency of the test vaccine in which, when the IQR of the test vaccine is larger than that of the reference, the potency of the test vaccine should be considered smaller than that of the reference, rather than vice versa. The conformity between the single-dose mouse protection test, the serological potency assay and immune efficacy in dogs was assessed by comparing with quality standards of test vaccines.

RESULTS

Antibody responses to different vaccines in the serological potency assay

7 groups (10 mice / group) were respectively immunized with different vaccines, and then the antibody titers of serum samples were measured by the FAVN test. As shown in Table 2, on the 14th day after vaccination, the median (20.58 IU/mL) of rabies-neutralizing antibody titers of group Vac 1 exceeded reference 3 (15.64 IU/mL), and the medians of group Vac 2, Vac 3 and Vac 4 were semiquantitated as being between reference 2 (3.01 IU/mL) and reference 3 (15.64 IU/mL). As a result, the potencies of the four test vaccines were in accord with their required qualities.

Conformity between the mouse protection test and the serological potency assay

To evaluate the accuracy and repeatability of the serological potency assay-based FAVN, repeat tests (3 times) and a single-dose mouse protection test were performed. As shown in Table 3, all results of repeat tests by the serological potency assay and of the single-dose mouse protection test accorded with the required qualities of test vaccines.

Humoral immune responses against rabies in dogs

The antibody titers of serum samples assayed by FAVN test on 14th day after vaccination are shown in Table 4, and the potency of test vaccines was evaluated by comparison of the protective efficacy of the test and reference vaccines in dogs. The median titer (6.15 IU/mL) of the rabies-neutralizing

Table 2. Antibody responses to different vaccines in serological potency assay

Groups	Antibody titers（IU/mL）of each serum sample										Md (IQR)*
	1	2	3	4	5	6	7	8	9	10	
Reference 1 (1 IU/mL)	1.14	0.10	1.14	0.50	0.87	0.42	0.38	3.42	0.42	0.87	0.69 (0.73)
Reference 2 (2 IU/mL)	3.42	7.79	0.50	5.92	0.87	4.50	2.60	4.50	1.14	0.66	3.01 (4.04)
Reference 3 (3 IU/mL)	2.60	5.92	17.77	7.79	30.77	17.77	23.38	30.77	13.50	7.79	15.64 (17.91)
Vac 1	2.60	90.32	17.77	7.79	90.32	30.77	23.38	53.30	13.50	7.79	20.58 (54.77)
Vac 2	30.77	7.79	1.14	4.50	17.77	0.42	0.29	13.50	7.79	13.50	7.79 (13.61)
Vac 3	17.77	3.42	0.87	1.97	23.38	2.60	4.50	1.14	7.79	5.92	3.96 (8.52)
Vac 4	7.79	0.66	3.42	0.66	13.50	10.26	0.87	3.42	0.38	4.50	3.42 (7.75)

* The median (Md) and interquartile range (IQR).

antibody of the group Vac 1 was equal to that of the group reference 3; however, the IQR (7.34 IU/mL) of Vac 1 was less than that (8.20 IU/mL) of reference 3; i.e., the protective efficacy of Vac 1 in dogs was higher than that of reference 3 (6.15 IU/mL). The protective efficacy of Vac 2, Vac 3 and Vac 4 in dogs was estimated to be between reference 2 (1.32 IU/mL) and reference 3 (6.15 IU/mL); i.e., the protective efficacy of the test vaccines in dogs was in line with their required potency.

Table 3. Conformity between single-dose mouse protection test and serological potency assay

Groups	Serological potency assay (IU/mL)			Single-dose mouse protection test (IU/mL)
	Test 1	Test 2	Test 3	
Vac 1	>3	>3	>3	3.08
Vac 2	2-3	2-3	2-3	2.87
Vac 3	2-3	2-3	2-3	2.34
Vac 4	2-3	2-3	2-3	2.11

DISCUSSION

As described here, the serological potency assay, based on the FAVN test, is a semi-quantitative and rapid method for testing the potency of inactivated rabies vaccines. The FAVN test is a reliable method recommended by OIE (World Organization for Animal Health), and shows good reproducibility and accuracy.

In general, the potency requirements of inactivated rabies vaccines is determined as no less than 1 IU/dose or 2 IU/dose (potency requirements). The NIH test and single-dose mouse protection test are widely used in vaccine manufacturing for the potency determination of semi-finished and finished products[18,24,26], in which more than one hundred mice are challenged with rabies virus, with the whole process taking at least one month to complete. However, depending on the required potency (≥1 IU/dose or ≥2 IU/dose) of inactivated rabies vaccines, a semi-quantitative assessment (e.g. >1 IU/dose or >2 IU/dose) of the potency is enough for vaccine quality control.

In the serological potency assay, only 10 mice are used for immunization with each test and reference vaccine. Use of the serological assay instead of the challenge test, therefore, reduced animal numbers by almost 90%. In most cases, the titers of antibody reach a peak by the 14th day after vaccination [15], on which day blood samples are collected from the retro-orbital sinus of mice for the FAVN test. Therefore, the serological assay requires shorter time than the NIH test and single-dose mouse protection test.

Currently, no locally produced veterinary inactivated vaccine against rabies in the Chinese market is available, and only 4 different brands of imported inactivated vaccines (Nobivac® Rabies, Rabvac™ 3, Rabisin-R and Rabigen®mono) have been approved by the Chinese government. These veterinary vaccines usually contain adjuvant (e.g., alum), whereas reference vaccines do not. In the NIH test and single-dose mouse protection test, the test vaccines are serially diluted, and so the action of adjuvants in vaccines will be reduced because of their low concentrations after dilution. Additionally, the potency of adjuvanted vaccines (antigen and adjuvant) would not be measured correctly by challenge tests. However, in our serological assay, test vaccines were not diluted before testing.

Some data have demonstrated that the routes and dosage of administration can have a significant impact on the immune efficacy of vaccines in mice[15,28]. The EP recommends intraperitoneal injection for the challenge test, and intramuscular or subcutaneous routes for the serological assay. In our experience, the intramuscular route is easier to perform for small volume (50 μL) vaccination than subcutaneous and intraperitoneal injection. We have found that 50 μL of one dose of adjuvanted vaccine for dogs is sufficient for the adult mouse immune system and can be injected completely in the back side of the upper hind leg. Less than this amount or more than the amount would produce non-detectable or excessive amounts of antibody, which would affect the judgment for the result (data not shown).

In early studies, a correlation has been found to exist between rabies-neutralizing antibody titers and protection from intracerebral challenge in mice[7,29]. The titer of rabies-neutralizing antibody is not the only parameter of immune response, but is the most reliable indicator of immune protection. We have observed that titers of antibody induced by vaccines in mice do not follow a normal distribution, which might be due to a different

Table 4. Humoral immune response in dogs vaccinated with different potency vaccines

Groups	Antibody titers assayed by FAVN test (IU/mL)						Md (IQR)*
	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5	Dog 6	
Reference 1 (1 IU/mL)	0.50	0.29	1.14	0.22	0.66	0.87	0.58 (0.67)
Reference 2 (2 IU/mL)	0.50	1.97	3.42	0.66	1.50	1.14	1.32 (1.71)
Reference 3 (3 IU/mL)	4.50	0.66	7.79	1.14	13.50	7.79	6.15 (8.20)
Vac 1	4.50	10.36	7.79	7.79	0.87	1.14	6.15 (7.34)
Vac 2	3.42	0.87	0.50	3.42	7.79	1.14	2.28 (3.74)
Vac 3	1.14	1.97	3.42	0.66	7.79	0.87	1.56 (3.40)
Vac 4	0.66	1.14	2.60	3.42	1.50	0.87	1.50 (1.79)

* The median (Md) and interquartile range (IQR).

out-bred species, i.e., Kunming mice, a widely used mouse breed in medical experiments in China, and therefore the median (Md) and interquartile ranges (IQR) were used to represent the immune efficacy of reference and test vaccines instead of the mean plus standard deviation[10]. By comparing the Md and IQR of antibody titers induced by reference and test vaccines, we can conclude whether or not the potency of the testing vaccines reaches or exceeds the potency requirement. To confirm the accuracy and repeatability of this serological potency assay, repeated tests, the single-dose mouse protection test and dogs vaccination were performed, and the results of the single-dose mouse protection test and immunization in dogs were compared with those of the serological potency assay. Our results showed that the immune efficacy in mice and dogs had high conformity with the potency assayed by the single-dose mouse protection method.

However, the antibody titers in individual mice varied significantly from 0.29 to 30.77 IU/mL for a given vaccine. Although we adapted the median (Md) and interquartile range (IQR) of serum antibody titers of each group, these variations should still influence the actual efficacy values of the vaccine, which is most likely due to the *in vivo* variation of immune responses to the vaccines. Therefore, an *in vitro* assay which determines the antigenic content (the trimeric form of G) would be expected to develop only when the adjuvant effect on the amount of G protein is excluded.

In conclusion, the serological method based on the FAVN test on the antibodies produced by certain amount of reference vaccine and a test vaccine is rapid, efficient, low-cost, and in accordance with the immunogenicity induced in dogs, and therefore can be recommended for batch potency testing during inactivated rabies vaccine production.

References

1. **Bruckner L, Kihm U.** 1986. Alternatives for potency testing of veterinary vaccines: rabies vaccine as a model. **Dev Biol Stand**, 64: 93-97.
2. **Barth R, Diderrich G, Weinmann E.** 1988. NIH test, a problematic method for testing potency of inactivated rabies vaccine. **Vaccine**, 6(4): 369-377.
3. **Cliquet F, Aubert M, Sagne L.** 1998. Development of a fluorescent antibody virus neutralization test (FAVN test) for the quantitation of rabies-neutralizing antibody. **J Immunol Mthods**, 212(1): 79-87.
4. **Ferguson M, Heath A.** 1992. Report of a collaborative study to assess the determination of glycoprotein antigen content of rabies vaccines for human use. **Biologicals**, 20: 143-154.
5. **Ferguson M, Schild G C**. 1982. A single-radial-immunodiffusion technique for the assay of rabies glycoprotein antigen: application to potency tests of vaccines against rabies. **J Gen Virol**, 59(1): 197-201.
6. **Fernández I, Peña A, Teso N D, *et al***. 2010. Clinical Biochemistry Parameters in C57BL/6J Mice after Blood Collection from the Submandibular Vein and Retroorbital Plexus. **J Am Assoc Lab Anim Sci**, 49(2): 202-206.
7. **Fitzgerald E A, Gallagher M, Hunter W S, *et al.*** 1978. Use of the antibody assay in immunized mice for the determination of rabies vaccine potency. **Dev Biol Stand**, 40: 183-186.
8. **Fournier-Caruana J, Poirier B, Haond G, *et al.*** 2003. Inactivated rabies vaccine control and release: use of an ELISA method. **Biologicals**, 31(1): 9-16.
9. **Gamoh K, Senda M, Itoh O, *et al.*** 1996. Use of ELISA for *in vitro* potency test of rabies vaccines for animal use. **Biologicals,** 24(2): 95-101.
10. **Gaddis G M, Gaddis M L**. 1990. Introduction to biostaticstics: Part 2, Descriptive statistics. **Ann Emerg Med**, 19(3): 309-315.
11. **Gamoh K, Shimazaki Y, Senda M, *et al***. 2003. Establishment of a potency test by ELISA for a rabies vaccine for animal use in Japan. **J Vet Med Sci**, 65(6): 685-688.
12. **Habel K.** 1996 Laboratory techniques in rabies: Habel test for potency. **Monogr Ser World Health Organ**, (23): 369-373.
13. **Hu R L, Fooks A R, Zhang S F, *et al.*** 2008. Inferior rabies vaccine quality and low immunization coverage in dogs (*Canis familiaris*) in China. **Epidemiol Infect**, 136(11): 1556-1563.
14. **Krämer B, Bruckner L, Daas A, *et al.*** 2010. Collaborative study for validation of a serological potency assay for rabies vaccine (inactivated) for veterinary use. **Pharmeur Bio Sci Notes**, 2010(2): 37-55.
15. **Krämer B, Schildger H, Behrensdorf-Nicol H A, *et al.*** 2009. The rapid fluorescent focus inhibition test is a suitable method for batch potency testing of inactivated rabies vaccines. **Biologicals**, 37(2):119-126.
16. **Lafon M, Perrin P, Versmisse P, *et al***. 1985. Use of a monoclonal antibody for quantification of rabies vaccine glycoprotein by enzyme immunoassay. **J Biol Stand**, 13: 295-301.
17. **Lindbladh C, Mosbach K, Bülow L**. 1993. Use of genetically prepared enzyme conjugates in enzyme immunoassay. **Trends Biochem Sci,** 18(8): 279-283.
18. **Monograph European Pharmacopoeia.** 1985. Vaccinum rabiei inactivatum ad usum veterinarium.In: **Monograph European Pharmacopoeia**, 2nd Ed. pp451.
19. **Mousli M, Turki I, Kharmachi H, *et al.*** 2007. Recombinant single-chain Fv antibody fragment-alkaline phosphatase conjugate: a novel *in vitro* tool to estimate rabies viral glycoprotein antigen in vaccine manufacture. **J Virol Methods**, 146(1-2): 246-256.
20. **Nagarajan T, Reddy G S, Mohana Subramanian B, *et al.*** 2006. A simple immuno-capture ELISA to estimate rabies viral glycolprotein antigen in vaccine manufacture. **Biologicals,** 34(1): 21-27.
21. **Nimmagadda S V, Aavula S M, Biradhar N, *et al.*** 2010. Recombinant diabody-based immunocapture enzyme-linked immunosorbent assay for quantification of rabies virus glycoprotein. **Clin Vaccine Immunol**, 17(8): 1261-1268.
22. **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.

23. **Rooijakkers E, Groen J, Uittenbogaard J, *et al.*** 1996. Development and evaluation of alternative testing methods for the *in vivo* NIH potency test used for the quality control of inactivated rabies vaccines. **Dev Biol Stand**, 86: 137-145.
24. **Seligmann EB Jr.** 1996. Laboratory techniques in rabies. Potency-test requirements of the United State National Institutes of Health (NIH). **Monogr Ser World Health Organ**, (23): 360-368.
25. **World Health Organization.** 2005. WHO Expert Consultation on Rabies. In: **First Report WHO Technical Report Series No. 931.** Geneva: World Health Organization. pp31-56.
26. **Wu X F, Hu R L, Zhang Y Z, *et al.*** 2009. Reemerging rabies and lack of systemic surveillance in People's Republic of China. **Emerg Infect Dis**, 15(8): 1159-1164.
27. **Wunderli P S, Dreesen D W, Miller T J, *et al.*** 2006. The rabies peripheral challenge test: more accurate determination of vaccine potency. **Vaccine,** 24: 7115-7123.
28. **Wunderli P S, Dreesen D W, Miller T J,** *et al*. 2003. Effects of vaccine route and dosage on protection from rabies after intracerebral challenge in mice. **Am J Vet Res**, 64(4): 491-498.
29. **Wunderli P S, Shaddock J H, Schmid D S, *et al.*** 1991. The protective role of humoral neutralizing antibody in the NIH potency test for rabies vaccines. **Vaccine,** 9: 638-642.