

Article @ Virology**Current Status and Challenges of Alternative Methods for Potency Testing of Human Rabies Vaccines**

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ABSTRACT

Rabies is a fatal viral disease, and vaccination is an effective means of prevention. Potency testing of rabies vaccines is crucial for ensuring vaccine quality. Currently, the NIH method is widely regarded as the gold standard for rabies vaccine potency testing; however, this method has limitations, including complexity, time consumption, and ethical concerns regarding animal use. In recent years, research in this field has gradually shifted towards alternative technologies aimed at improving testing efficiency, reducing costs, and addressing animal welfare requirements. This article reviews the research progress of various alternative technologies based on vaccine antigen detection and antibody detection, including enzyme-linked immunosorbent assay, time-resolved methods, rapid fluorescent focus inhibition test, and antibody binding test. It systematically analyzes the principles, technical limitations, and feasibility of these methods as alternatives in vaccine quality control, aiming to provide new insights and theoretical foundations for rabies vaccine potency testing to promote further development in this field.

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Article History: Submitted: 28/01/2025; Revised: 10/02/2025; Accepted: 21/02/2025

DOI: 10.21092/jav.v14i1.117

Key Words: Rabies Vaccine, Potency Testing, NIH method, Method Alternatives, Antigen Detection, Antibody Detection

Abbreviations: RABV, Rabies Virus; NIH method, method established by the National Institutes of Health; ELISA, Enzyme-linked Immunosorbent Assay; RFFIT, Rapid Fluorescent Focus Inhibition Test; ABT, Antibody Binding Test; TRFIA, Time-resolved Fluorescence Immunoassay.

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Introduction

Rabies is a fatal zoonotic disease, and vaccination is the most effective means of preventing and controlling it. Currently, the NIH method is the gold standard for potency testing of human rabies vaccines, but this method has obvious limitations such as long experimental cycles, complex operations, and high animal usage. With the development of biotechnology and increasing attention to animal welfare, finding reliable and efficient alternative methods has become an important research direction in the field of vaccine quality control.

This article aims to comprehensively examine the advantages and disadvantages of the current NIH method and systematically evaluate the research progress and application prospects of alternative technologies based on antigen detection and antibody detection, providing scientific references for the quality control of rabies vaccines.

As the requirements for vaccine quality control continue to rise, research on alternative testing methods for rabies vaccine potency has gradually increased, especially various *in vitro* testing technologies based on antigen or antibody detection. For example, one study proposed a new antibody binding test protocol for assessing the potency of rabies vaccines. The principle of this experiment is to combine the rabies vaccine with a quantitative neutralizing antibody

against rabies virus, and then combine it with a quantitative fluorescently labeled rabies virus. By calculating the remaining fluorescent area using fluorescence observation equipment, the vaccine potency is calculated using the Reed-Muench method. This study showed that the potency testing results of this method for multiple batches of rabies vaccine final products were consistent with the NIH method, demonstrating its potential as an alternative testing method^[1].

Another study evaluated the potency of seven commercially available veterinary rabies vaccines in Sri Lanka using a mouse challenge test. The results showed that some single-dose vaccines had a potency of less than 1.0 IU/dose, failing to meet standards, emphasizing the importance and necessity of potency assessment before vaccine market release^[2].

The double antibody sandwich ELISA, as an antigen detection-based *in vitro* immunological technique, is seen as a potential replacement for the NIH method in recent studies. This method uses microtiter plates coated with neutralizing antibodies (RAB1) to detect the rabies glycoprotein antigen, and results indicate that this method outperforms traditional methods in terms of accuracy, precision, and sensitivity, effectively distinguishing between high and low potency vaccine samples. Studies have shown a certain correlation between this method and NIH potency values (Pearson's $r=0.81$), thus providing an effective

alternative for rabies vaccine quality control^[3].

Despite the dominant position of the NIH method in rabies vaccine potency testing, its obvious technical and ethical limitations have prompted researchers to actively explore more reliable alternative methods. Currently, various novel technologies based on antibody binding and antigen detection are gradually becoming a powerful supplement to vaccine quality control. These methods not only improve testing efficiency and reduce animal usage (3R principles) but also provide more reliable guarantees for vaccine market release and application. Future research should continue to refine these alternative methods to meet the evolving public health needs and animal welfare standards.

Current Status and Limitations of the Gold Standard NIH Method

1. Basic Principles and Standardized Operating Procedures of the NIH Method

The NIH method was first established by researchers at the National Institutes of Health in the United States, so it is also referred to as the NIH method. The NIH method (National Institutes of Health Method) is currently the gold standard for assessing the potency of human rabies vaccines, based on evaluating the survival rate of immunized animals (usually mice) after viral challenge. The operating process

is shown in Figure 1. The experimental design of this method includes the selection of animal models, the formulation of immunization protocols, and the implementation of challenge tests.

In the operating procedure, an appropriate animal model is first selected, and the vaccine is injected into the animals according to the specified immunization protocol. Subsequently, the animals are challenged with the virus to observe their survival, thereby assessing the vaccine's effectiveness. Standardization organizations, both international and domestic, such as the World Health Organization (WHO) and the United States Department of Agriculture (USDA), have strict standards for the NIH method, including vaccine purity, active ingredient content, and stability. These standardized operating procedures ensure the reproducibility of experiments and the reliability of results, which are crucial for the quality control of rabies vaccines^[4].

2. Advantages of the NIH Method in Vaccine Quality Control

As the currently widely used gold standard method, the NIH method has a solid historical foundation and scientific basis, having been widely adopted globally for a long time. This method not only provides scientific validation for the immunoprotective effects of vaccines but also quantitatively reflects the *in vivo* protective efficacy of vaccines through animal survival rates.

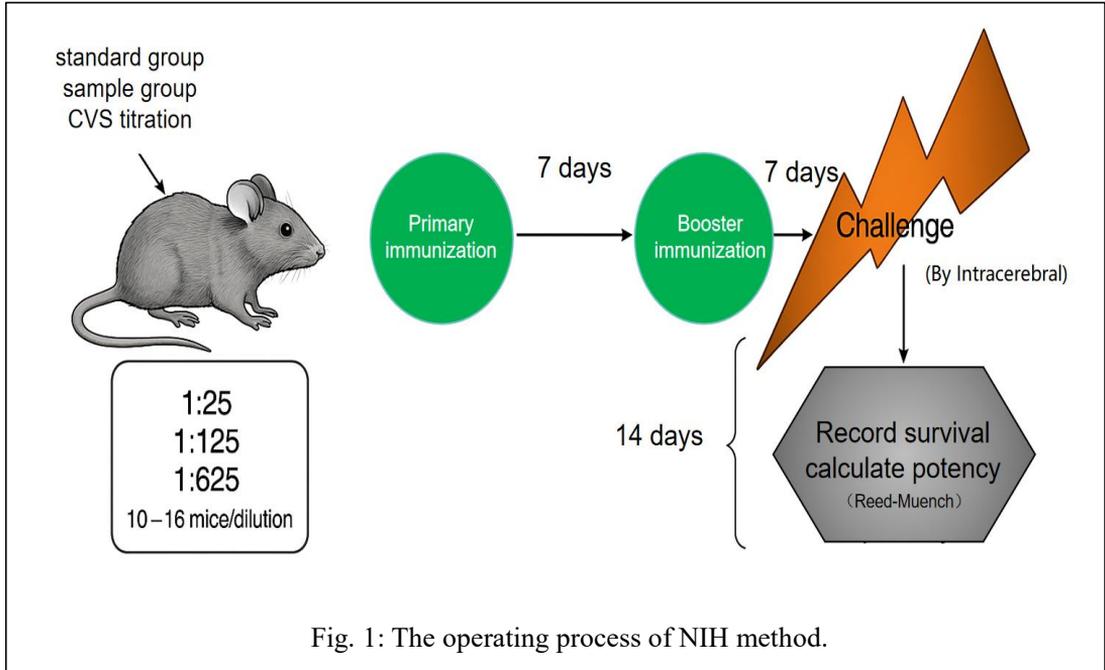


Fig. 1: The operating process of NIH method.

Multiple studies and historical data indicate that the NIH method can reliably predict vaccine performance in actual applications. Furthermore, the standardized operating procedures of the NIH method ensure that results from different laboratories are comparable, providing a solid foundation for vaccine quality control. This reliability boosts public confidence in rabies vaccines and supports the development of global public health policies^[5].

3. Major Limitations of the NIH Method

Despite its important role in vaccine evaluation, the NIH method's technical and application limitations have become increasingly prominent with the accelerated pace of vaccine development and tightening ethical regulations^[6,7].

First, the NIH method requires a large number of mice for each batch of vaccine, raising significant ethical concerns and involving substantial consumption of animal resources and costs, conflicting with the internationally advocated '3R principles'^[8].

Second, the experimental cycle of the NIH method is relatively long, typically requiring 4-5 weeks, which constrains the batch release of vaccines, especially in response to public health emergencies, easily delaying the vaccine supply chain response^[9].

Additionally, the NIH method suffers from issues such as high variability in experimental results and poor reproducibility, influenced by multiple factors including the strain of experimental animals, the condition of experimental animals, the experience of

operators, and the virulence of the virus, leading to poor reproducibility and data stability. These limitations have become the core driving force for the development of alternative methods, prompting researchers to focus on establishing a more efficient, objective, and ethically compliant rabies vaccine potency evaluation system^[10].

Research Progress on Alternative Methods for Potency Testing

1. Research Progress on Alternative Technologies Based on Vaccine Antigen Detection

1.1 Development and Application of ELISA

In the development of rabies vaccines, enzyme-linked immunosorbent assay (ELISA) has been widely used for in vitro detection of antigen content because of its specificity and sensitivity. In establishing a specific ELISA for rabies virus glycoprotein, suitable monoclonal antibodies must first be screened as capture antibodies^[11].

Research has found that high purity and active glycoprotein can be obtained through recombinant expression of rabies virus glycoprotein, which can then be used to draw the standard curve for ELISA. By comparing with known concentration standards, the detection conditions of ELISA, such as antibody concentration, incubation time, and reaction temperature, can be optimized to achieve sensitive detection of rabies virus glycoprotein. This method has

good specificity and accuracy, effectively distinguishing different amounts of antigen^[12].

The European EPAAA organization once initiated an international collaborative study on NIH alternative methods, which showed that the relative potency detected using the established ELISA method was comparable to that of the NIH method, and this ELISA alternative method could detect heat-inactivated glycoproteins, i.e., conformational glycoprotein antigen content^[13].

Additionally, some scholars have used competitive inhibition ELISA to evaluate the in vitro potency of human rabies vaccines, and this method has been validated to comply with ICH guidelines, allowing for the assessment of heat-degraded vaccines, with good correlation in the comparative analysis of 25 batches of human rabies vaccine potency^[14].

1.2 Time-Resolved Fluorescence Immunoassay Technology

Time-resolved fluorescence immunoassay (TRFIA) technology shows significant advantages in detecting vaccine antigens thanks to its remarkable sensitivity and specificity. The working principle of TRFIA is based on using antibodies labeled with rare earth metal ions that have long fluorescence lifetimes, suppressing background fluorescence through time-resolved technology, thereby improving the signal-to-noise ratio. An important breakthrough of this technology is its

enhancement in detection sensitivity; studies have shown that TRFIA can detect antigen concentrations as low as pg/ml, which is difficult to achieve with traditional ELISA methods, making it suitable for high-sensitivity vaccine potency assessment scenarios (see figure 2).

With its high sensitivity and specificity, TRFIA is regarded as one of the key technologies with great potential in future vaccine antigen detection systems. Research has utilized TRFIA to detect rabies antigen content and compare it with vaccine potency, showing a correlation coefficient of 0.903 between the potency values obtained by TRFIA and the NIH method^[15].

1.3 Challenges in Standardizing Antigen Detection Methods

Although antigen detection methods have wide applications in vaccine monitoring, there are still many technical bottlenecks in standardization and the process of replacing the traditional NIH method.

First, the difficulty in aligning results between methods is a significant challenge currently faced. While methods like ELISA demonstrate good reproducibility and stability within laboratories, the NIH method itself has high variability in results due to individual differences among animals and fluctuations in experimental conditions, making it difficult to establish a stable correspondence between the two methods. Therefore, the quantitative results of antigens are difficult to accurately map to NIH potency readings, affecting mutual recognition between the two methods.

Secondly, the differences in antigen conformation between strains further complicate standardization. The core principles of ELISA and TRFIA methods are to recognize specific spatial epitopes of antigens, thus requiring high stability of the conformations of antigen epitopes. However, there are significant differences in the spatial conformations and glycosylation patterns of

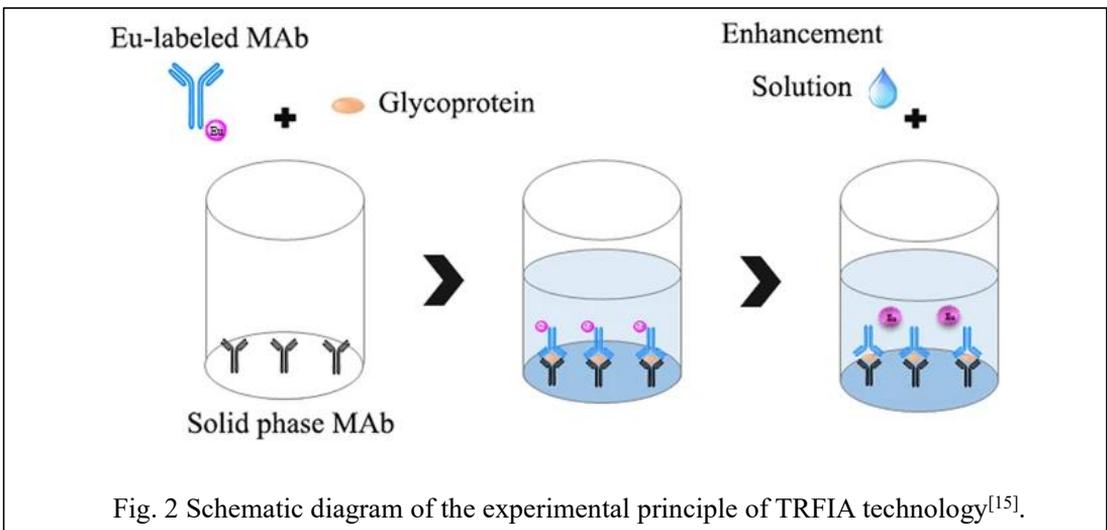


Fig. 2 Schematic diagram of the experimental principle of TRFIA technology^[15].

glycoproteins among different rabies virus strains, making it challenging for a single antibody to be uniformly applicable for quantitative detection of all vaccine strains, leading to the potential for underestimating antigen quantities in detection results^[16-18].

Moreover, high-sensitivity detection technologies like TRFIA are highly dependent on experimental conditions (such as fluorescence stability, background suppression, and reaction time), and differences in equipment, fluorescent matrices, and operational techniques among different laboratories lead to difficulties in standardization during their promotion. Additionally, the detection signal of TRFIA is based on instantaneous fluorescence readings within a time window, which has low tolerance for changes in antigen conformation and concentration, limiting its applicability in complex samples^[19].

In summary, although antigen detection methods have advantages such as ease of operation and no need for animals, they still face significant technical bottlenecks in spatial recognition consistency, functional predictive capability, inter-method conversion, and inter-laboratory reproducibility. Therefore, before considering them as alternatives to the NIH method, it is necessary to comprehensively assess vaccine quality in conjunction with antibody functional validation (such as RFFIT) to bridge the gap between "detection quantity" and "protective efficacy."

2. Research Progress on Alternative Technologies Based on Antibody Detection

2.1 Rapid Fluorescent Focus Inhibition Test

The rapid fluorescent focus inhibition test (RFFIT) is the standard method for assessing the neutralizing antibody titer against rabies virus. The methodology is based on traditional virus neutralization tests, which have been optimized multiple times to improve efficiency and accuracy.

Traditional RFFIT tests are relatively complex, involving multiple steps such as cell culture, virus infection, antibody treatment, and color development. To enhance detection efficiency and reliability, researchers have introduced recombinant rabies viruses (such as rRV-GFP), allowing the detection process to be conducted by directly observing green fluorescence, thus shortening detection time and improving experimental convenience and accuracy.

Related studies have shown a good correlation between RFFIT and the traditional NIH method, with a Pearson correlation coefficient as high as 0.9776, indicating consistent results in detecting neutralizing antibodies against rabies virus, especially in samples close to the minimum vaccine potency threshold (0.5 IU/ml)^[20].

Therefore, RFFIT is gradually being recognized as an effective alternative method for rabies vaccine potency testing.

2.2 Antibody Binding Test (ABT)

The antibody binding test (ABT) is a method that assesses rabies vaccine potency

by quantitatively detecting the binding ability of antibodies to antigens. Its principle involves combining the rabies vaccine with a known concentration of neutralizing antibodies against rabies virus, and then the remaining rabies vaccine binds with a quantitatively fluorescently labeled rabies virus. The unbound fluorescently labeled rabies virus is measured using fluorescence observation instruments, and the fluorescent area is calculated, with the potency of the rabies vaccine determined using the Reed-Muench method.

Testing results for multiple batches of rabies vaccine raw materials and finished products indicate that the potency measured by this method is consistent with the results of the NIH method. The advantage of this method lies in its potential for high-throughput screening, enabling a large number of vaccine samples to be screened for potency in a shorter time, significantly enhancing testing efficiency and laboratory productivity^[1].

2.3 Standardization Research of Antibody Detection Methods

The standardization of antibody detection methods is a key step in achieving comparability of results across different laboratories and improving the reliability of methods. With the establishment of international standards, the accuracy and reliability of antibody detection have significantly improved. For example, the World Health Organization (WHO) has

established international standards for rabies immunoglobulin as a benchmark for antibody measurement. The application of this standard allows laboratories in various countries to refer to the same standard when conducting rabies antibody detection, thereby reducing result discrepancies caused by differences in experimental conditions and operational methods.

However, achieving complete comparability of results among different laboratories still faces challenges, including variations in antigen sources, differences in experimental equipment, and the experience of operators, all of which may affect the final detection results. In recent years, research addressing these issues has gradually increased, aiming to resolve these challenges by optimizing experimental processes and enhancing the uniformity of detection methods^[21].

Key Scientific Points in Validating Alternative Methods

When evaluating the correlation of alternative methods with the traditional NIH method, several key scientific issues must be considered to ensure that the alternative methods possess the same scientific validity and practicality as the traditional NIH method.

First, an appropriate sample size and experimental design are fundamental for validating alternative methods. Validation of alternative methods typically involves statistical correlation analysis with the

results of the NIH method, thus requiring a pre-determined reasonable sample size to achieve sufficient statistical power. A sample size that is too small will not yield robust validation results, while a sample size that is too large will result in unnecessary animal use and resource waste.

Second, alternative methods should have a correlation with the mechanism of action of the traditional NIH method. The core principle of the NIH method is to assess the protective effect of the vaccine against viral attack *in vivo*; therefore, it is more persuasive if alternative methods are designed based on similar immunological principles. For example, the rapid fluorescent focus inhibition test (RFFIT), which directly detects neutralizing antibodies induced by the vaccine, aligns closely with the immune protection mechanism of the NIH method; in contrast, antigen detection methods like ELISA primarily measure antigen content, which has a more indirect correlation with protective efficacy and should be applied cautiously^[20].

Furthermore, the precision and accuracy of alternative methods must match those of the NIH method, with particular attention to the standardization and reproducibility of alternative methods. Precision reflects the stability of the method itself, while accuracy determines whether the method reliably predicts the protective efficacy of the vaccine. These two indicators not only

determine whether alternative methods can be accepted by the scientific community but are also critical for regulatory agencies in evaluating whether alternative methods can be applied to actual vaccine batch release. At the same time, the determination and standardization of key technical parameters such as limit of detection (LOD) and limit of quantification (LOQ) directly affect the reliability and sensitivity of alternative methods in practical applications.

Finally, the differences in vaccine production processes and strains among countries cannot be ignored when selecting alternative methods. In Europe and the United States, rabies vaccines are typically produced using a single standard strain, resulting in stable and consistent antigen conformations, making antigen quantification-based ELISA methods suitable. This method is not only simple and rapid but also easy to standardize and promote. However, for countries like China, where there are various vaccine production strains and processes, significant differences in glycoprotein epitopes among different strains make it difficult to establish a unified standard for ELISA; in this case, antibody detection methods that directly reflect vaccine immune effects are more appropriate, as they reflect the neutralizing capacity *in vivo*.

Moreover, practical factors such as animal welfare, regulatory policies, economic costs, laboratory infrastructure, and personnel

operational skills will also significantly impact the actual promotion and application of alternative methods. Therefore, in the process of validating alternative methods, in addition to scientific rigor and technical indicators, it is also necessary to fully consider practical application scenarios and national differences, constructing a diversified, flexible, and adaptive vaccine potency testing scheme to achieve the scientific promotion and effective application of alternative methods globally.

Conclusion

Standardizing rabies vaccine potency testing methods and developing alternative methods are key research topics in the field of vaccine quality control. For a long time, the NIH method, centered on animal challenge, has been widely used globally, but it has obvious limitations, including high animal usage, long experimental cycles, and high variability in results. These shortcomings not only bring ethical pressure but also limit the efficiency of vaccine batch release and the rapid response capability to public health emergencies.

In recent years, with continuous advancements in science and technology, significant progress has been made in research on alternative testing methods for rabies vaccine potency. In particular, antigen detection-based ELISA and TRFA, as well as antibody detection-based RFFIT and ABT, have all shown promising application

prospects. The introduction of these methods provides effective alternatives to the traditional NIH method, promoting innovative development in rabies vaccine quality control.

The World Health Organization (WHO) has an open attitude towards alternative potency testing methods for rabies vaccines in its relevant technical documents, emphasizing the need to update potency testing standards based on the latest scientific evidence and technological advancements. WHO's technical guidelines indicate that traditional vaccine potency testing methods, such as neutralization tests, are complex and resource-intensive, particularly in resource-limited countries, making implementation challenging.

Therefore, WHO actively encourages researchers to develop and validate new *in vitro* alternative methods, including ELISA, RFFIT, and other faster and more animal-friendly technologies. These alternative methods can not only improve testing efficiency but also reduce the requirements for laboratory facilities and technical personnel, making them more universally applicable in vaccination and monitoring. Alternative methods must undergo rigorous validation and comparison with traditional methods to ensure their effectiveness and reliability in practical applications.

In summary, the future development trend of alternative methods for rabies vaccine

potency testing should focus on strengthening the standardization and international recognition of the methods themselves, establishing alternative strategies that closely align with the mechanisms of action of the traditional NIH method, and formulating flexible and diverse evaluation systems based on the actual vaccine production and regulatory environments of different countries. Only in this way can the global promotion and application of alternative testing technologies be effectively advanced while ensuring vaccine quality and safety.

Competing interests

The authors declare all financial and non-financial competing interests.

Funding

The work is supported by the State Key Laboratory of Drug Regulatory Science Project (Grant No. 2023SKLDRS0111) .

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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