RESEARCH ARTICLE

Establishment and Application of a Direct Immunofluorescence Assay for Determining Rabies Virus Titer

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ABSTRACT

Objective: To establish and validate a detection method for the titration of extracellular rabies virus as an alternative to the intracerebral mouse titration method.

Methods: The direct immunofluorescence method was developed using Vero cells and BHK-21 cells to determine the virus titer of the rabies virus (CTN strain) in vaccine harvest fluid. The precision, relative accuracy, and linear range of the method were validated.

Results: Both cell types exhibited distinct fluorescent infection foci within 24 hours, with the number of infections decreasing as the virus was diluted. This observation indicates the method's applicability, with Vero cells demonstrating greater sensitivity than BHK-21 cells. Method validation revealed that the relative standard deviations (RSD) for repeatability and intermediate precision were both less than 15%. Additionally, the relative bias (RB) for relative accuracy was below 10%. The virus dilution factor, ranging from 5^0 to 5^{-9}, displayed a strong linear relationship with the virus titer, further confirming the method's effectiveness.

Conclusion: The direct immunofluorescence method established using Vero cells and BHK-21 cells demonstrates significant potential for quantifying the virus in the harvest fluid of human rabies vaccines derived from Vero cells.

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Key Words: Rabies Virus, CTN-1V strain, Direct Immunofluorescence Assay, Virus Titer

Abbreviations: RABV, Rabies Virus; DFA, Direct Immunofluorescence Assay; ELISA, Enzyme-linked Immunosorbent Assay; RSD, Relative Standard Deviation.

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Introduction

Rabies is a disease caused by the rabies virus that can infect both humans and animals,and it is widespread globally.The most notable characteristic of this disease is that once symptoms appear,the mortality rate in patients approaches 100%[1]. In China and other developing countries,rabies remains a severe threat to both humans and domestic animals.The rabies virus is neurotropic in nature. Due to the protective myelin sheaths and connective tissues surrounding neural structures,even high concentrations of specific antibodies in the bloodstream cannot easily penetrate neural tissues to exert immunological effects.However,vaccination can stimulate active immunity in the host:the produced antibodies can neutralize viruses before they enter the nervous system,while T-cell immunity can eliminate virus-infected cells.Therefore,vaccination remains the only effective method for both preventing and treating rabies.

To date,the commonly used virus strains for human rabies vaccines in China include the aG,CTN-1V,PV,and PM strains,among others[2].Notably,the CTN-1V strain was isolated from a rabies patient in Shandong Province,China, making it particularly suitable for rabies prevention in the Chinese population[3]. In the production process of human rabies vaccines by various manufacturers,the quality of virus seeds and harvest fluids is typically assessed through virus titer determination.Traditionally,virus titer has been measured using the intracerebral mouse inoculation method. However,this method places high demands on laboratory personnel and is susceptible to variability due to individual differences among mice.Consequently,the results obtained through this animal-based approach are often inconsistent and lack reproducib--ility.Additionally,the method requires a prolonged experimental cycle,including a 14-day observation period.Given the demands of large-scale vaccine production, establishing a rapid method for determining rabies virus titer has become critically important.The World Health Organization (WHO) has incorporated the direct immunofluorescence assay (DFA) into rabies vaccine testing protocols.Domestic research has also demonstrated that DFA can effectively replace the intracerebral mouse titration method[4-9].

This study aimed to establish direct immunofluorescence assays (DFA) using BHK-21 and Vero cells respectively,as alternatives to animal-based methods,for determining the viral titer in harvest fluids of human rabies vaccines produced with the rabies virus (CTN-1V strain).

Materials and Methods

1. Virus and Cells

Rabies virus harvest fluid(batches: DR23001-B-02,DR23002-B-01) was provided by our company. Vero cells and BHK-21 cells were obtained from ATCC.

2. Reagents and Instruments

FITC-labeled rabies diagnostic antibody (Military Veterinary Research Institute, China); Acetone (Sinopharm Chemical Reagent Co.,Ltd.); Cell culture media (Zhong Sheng Ao Bang Biotechnology Co.,Ltd.), Fetal bovine serum (Lanzhou Rongye Biotechnology Co.,Ltd.), Trypsin (Gibco,USA); Inverted fluorescence microscope (Olympus, Japan).

3. Establishment of DFA

The rabies virus harvest fluid was serially diluted 10-fold in PBS.Aliquots (50 μL) of each viral dilution (10^{-1} to 10^{-6}) were added to a 96-well plate,followed by an equal volume of Vero cell suspension (5×10^6 cells/mL).The same procedure was repeated using BHK-21 cells instead of Vero cells.Each dilution was tested in octuplicate (8 replicate wells).The plates were incubated at 37℃ with 5% CO_2~ for 24 hours.

Post-incubation,the culture medium was aspirated and cells were washed three times with PBS.After removing the wash solution,cells were fixed with 80% cold acetone.Working concentration (1:200 dilution) of FITC-conjugated rabies diagnostic antibody was then added and incubated for 1 hour. Following three additional PBS washes,specific fluorescent foci were examined under a fluorescence microscope.

Control settings included: Negative controls: Virus-free Vero and BHK-21 cells. Positive control: Cell suspension containing undiluted rabies virus harvest fluid.

Wells displaying specific fluorescent foci were scored as positive.Viral titer was calculated using the Reed & Muench method.

4. Method Validation

4.1 Intermediate Precision Validation

Two independent operators performed virus titer determination on the same rabies virus harvest fluid using the direct immunofluorescence assay (DFA) established in Section “3.Establishment of DFA” .

Each operator repeated the assay three times at different time points.The relative standard deviation (RSD) of the virus titer results was calculated to assess intermediate precision.

4.2 Repeatability Validation

A single batch of rabies virus harvest fluid was tested by the same operator following

the DFA protocol (Section “3.Establishment of DFA”). Six replicate measurements were performed.The mean titer ($\bar{x}$),standard deviation (SD), and relative standard deviation (RSD) were calculated to evaluate repeatability.

4.3 Relative Accuracy Validation

A virus harvest fluid with a known titer (determined by the mouse intracerebral assay) was serially diluted five-fold in PBS containing 2% heat-inactivated newborn calf serum.Three replicates of dilutions (5^{-1} to 5^{-3}) were tested using DFA.

The theoretical titer (from the mouse assay) was compared with the measured titer (from DFA),and the relative bias (RB) between them was calculated.

4.4 Linearity Range

A batch of virus harvest fluid was subjected to five-fold serial dilutions (up to 5^{-9}).Ten samples (including the undiluted virus and diluted samples) were tested for viral titer.A standard curve was generated with sample concentration (logdilution factor) on the x-axis and log $CCID_{50}$ on the y-axis.The linear range of the method was determined,and the coefficient of determina--tion (R^2) was calculated.

5. Statistical Analysis

Data were analyzed using SPSS 18.0 software(free version).Intermediate precision results were compared using one-way ANOVA,while accuracy was assessed via linear regression analysis. A P-value<0.05 was considered statistically significant.

Results

1.Intermediate Precision

Two operators independently measured the viral titer of the same harvest fluid using both BHK-21 and Vero cells at different time points,with three replicates per test. The results are presented in table 1.

The relative standard deviations (RSD) were 7.4% (BHK-21 cells) and 11.3% (Vero cells),both below the 15% acceptability threshold. No statistically significant differ--ence was observed between the operators' results: BHK-21 cells: Independent t-test,P = 0.628; Vero cells: Independent t-test,P = 0.423; The assays established with both cell types demonstrated excellent intermediate precision.

2. Repeatability

The same operator performed six replicate tests on the same sample using both BHK-21 and Vero cells.As shown in table 2,the mean viral titers were 7.1 $lgCCID_{50}$/mL (BHK-21 cells) and 7.4 $lgCCID_{50}$/mL (Vero cells),with relative standard deviations (RSD) of 8.4% and 10.7%, respectively.

These results demonstrate good repeatability for both cell lines.

3. Relative Accuracy

For the viral harvest fluid with a theoretical titer of 7.9 lgCCID$_{50}$/mL (as determined by the reference method),the same analyst performed viral titer determina--tions using both BHK-21 and Vero cells after serial dilution.The relative bias (RB) values ranged from 5.2% to 8.6%,all below the 10% acceptance criterion, demonstrating good method accuracy (see table 3).

Table 1: Intermediate precision validation results (lgCCID$_{50}$/mL)

Cell Line	Operator	Test Results	Intra-assay RSD（%）	Inter-assay RSD（%）	Statistical Analysis (p-value)
BHK-21 cells	Operator 1	7.5 6.6 6.8	6.8	7.4	0.628
	Operator 2	6.6 7.4 6.2	9.1		
Vero cells	Operator 1	6.4 7.4 6.8	7.3	11.3	0.423
	Operator 2	7.5 6.4 8.5	14.1		

Table 2: Repetitive verification results (lgCCID$_{50}$/mL)

Replication	BHK-21 cells			Vero cells		
	lgCCID$_{50}$/mL	$\bar{x}\pm s$	RSD（%）	lgCCID$_{50}$/mL	$\bar{x}\pm s$	RSD（%）
1	7.8	7.1±0.6	8.4	8.0	7.4±0.8	10.7
2	7.4			7.2		
3	6.6			7.5		
4	7.7			6.7		
5	6.8			8.5		
6	6.4			6.4		

Table 3: Relatively accurate validation results ($lgCCID_{50}$/mL)

Replication	BHK-21 cells			Vero cells		
	5^{-1}	5^{-2}	5^{-3}	5^{-1}	5^{-2}	5^{-3}
Theoretical Value	7.2	6.5	5.8	7.2	6.5	5.8
1	7.0	6.3	5.3	7.8	7.2	6.1
2	7.9	7.1	6.4	7.1	6.5	5.8
3	8.2	7.5	6.5	8.3	7.4	6.9
Mean Value	7.7	7.0	6.1	7.7	7.0	6.3
RB（%）	6.9	7.7	5.2	6.9	7.7	8.6

4. Linearity Range

The standard curve for the direct immuno--fluorescence assay (DFA) using BHK-21 cells is shown in figure 1(the left). A strong linear correlation (R^2 = 0.9595) was observed between viral titer and dilution factor across the range of 5^0 to 5^{-9}, with the linear regression equation Y = -0.5758x + 7.1509. Similarly, the DFA standard curve for Vero cells (figure 1, the right) demon--strated excellent linearity (R^2 = 0.994) over the same dilution range (5^0 to 5^{-9}), with the regression equation Y=-0.6376x+7.4691. Both cell lines achieved R^2 values >0.95, confirming the method's satisfactory linear performance within the tested range.

Discussion

Following exposure through wounds,the rabies virus exhibits significant individual variability in its incubation period. While most cases manifest within 1~3 months, epidemiological surveillance has document--ed rare instances of prolonged incubation exceeding 12 months. Upon symptom onset, patients develop progressive central nervous system dysfunction, characterized by patho--genic clinical features including hydrophobia, aerophobia (abnormal sensitivity to air currents), and photophobia (aversive response to light stimuli). Currently, no effective interventions exist once the virus enters the replication phase within the CNS,underscoring the critical

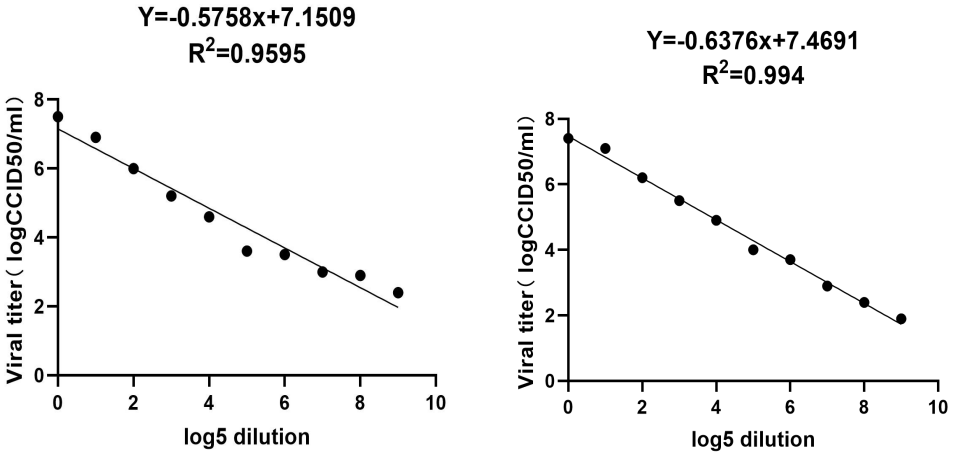

Figure1: Standard curve of the rabies virus titer determination method established in BHK-21 cells(left) and Vero cells

importance of timely post-exposure prophylaxis (PEP) with rabies vaccines and immunoglobulins[10].

As the primary preventive measure, vaccine quality is paramount-particularly its viral antigen content.This significance stems from the glycoprotein spikes on the viral surface,which serve as the sole protective antigen capable of inducing neutralizing antibodies in hosts [11-12]. Consequently, precise quantification of viral titer during vaccine manufacturing represents the most critical quality control parameter.

The Chinese Pharmacopoeia (2020 Edition, Volume III) mandates intracerebral mouse titration for rabies virus harvest fluid testing during production [13].This method relies on measuring the 50% lethal dose (LD_{50}) through viral proliferation in mouse brains and subsequent mortality monitoring. Although reliable,this assay suffers from Prolonged duration, Technical complexity, Inter-individual variability , Poor reproduci--bility and and requires substantial animal use,which conflicts with animal welfare principles.

This study employed a direct immuno--fluorescence technique (DFA) based on viral propagation in cell culture.Fixed cells were incubated with fluorescein-labeled specific antibodies that bind to rabies virus antigens,with subsequent fluorescence

excitation under UV/blue-violet light (emitting yellow-green fluorescence) for viral quantification.Compared with the traditional intracerebral mouse titration method,this approach significantly shortens the testing duration,eliminates the poor reproducibility caused by inter-mouse variability,and substantially reduces time costs in vaccine production processes.

Method validation demonstrated excellent precision and accuracy for the DFA.The CTN-1V strain showed strong linear correlation (R^2 >0.95) between viral titer and dilution factors (5^0 to 5^{-9}),with Vero cells exhibiting superior sensitivity (R^2 =0.994) versus BHK-21 cells,indicating enhanced linearity for this cell line.

In summary,the direct immunofluoresc--ence assay (DFA) effectively replaces the intracerebral mouse titration method, significantly reducing both experimental duration and costs while maintaining result accuracy and substantially decreasing animal usage,aligning with international trends in animal protection[14-16]. This method enables high-throughput sample detection in a single experiment,dramatically improving testing efficiency.Furthermore,with the ongoing refinement of laboratory animal welfare regulations,adherence to the 3Rs principles (Replacement,Reduction,and Refinement) is essential to minimize animal use and promote alternative methods[17].Therefore,we conclude that establishing DFA for determining the viral titer of CTN-1V strain represents a feasible approach.

Competing interests

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

References

[1] Zhang YZ,Yu YX,Dong GM,et al. Epidemio--logical characteristics of rabies in China and recommendations for its prevention and control[J].Chinese Journal of Preventive Medicine,2007,41(3):165-168.

[2] Shi LT,Li YH.Research and application progress of Vero cells in human rabies vaccines[J]. Chinese Journal of Zoonoses,2022, 38(03):260-265.

[3] Shi LT,Yu YX,Liu JH,et al.Genomic sequence analysis of Chinese rabies vaccine strain CTN-1[J].Virologica Sinica,2010,26(3):195-201.

[4] Li YY,Peng XH,Zhang S,et al.Optimization and validation of direct immunofluorescence assay for rabies virus titration[J].International Journal of Biologicals,2023,46(5):258-262.

[5] Liu HW,Tian YL,Zhu KY,et al.Establishment and validation of direct immunofluorescence assay for rabies virus titration[J].Chinese Journal of Biologicals,2021,34(6):721-724.

[6] Zhou LZ,Liu YD,Dai ML,et al.Fluorescent focus assay for rabies virus titration[J].Chinese Journal of Biologicals,2015,28(10):1092-1096.

[7] Wang MS,Zheng Y,Guo LS,et al.Optimization and validation of conditions for direct immunofluorescence assay in rabies virus titration[J].Chinese Journal of Biologicals, 2014,27(11):1477-1480.

[8] Tan XR,Chu XM,Zhang JB.Feasibility study of direct immunofluorescence assay for rabies virus titration[J].Progress in Microbiology and Immunology,2011,39(2):22-24.

[9] Chai M,Xu GL,Sun W,et al.Optimization of fluorescent focus assay for in vitro titration of rabies virus[J].Chinese Journal of Vaccines and Immunization,2010,16(3):258-260.

[10] SAMEH B ,BAKIR M ,OMAR B ,et al.Pathological study and detection of bovine parainfluenza 3 virus in pneumonic sheep lungs using direct immunofluorescence antibody technique [J].Compar Clin Pathol,2021, 8(2):10-11.

[11] Guo XY,Pan D,Chen XX,et al.Establishment and validation of double-antibody sandwich ELISA for detection of rabies virus glycoprotein antigen content[J].Chinese Journal of Biologicals,2020,33(4):434-444.

[12] De Rezende AG,Fernández Núñez EG,Astray RM,et al.An optimization study for expression of the rabies virus glycoprotein (RVGP) in mammalian cell lines using the Semliki Forest virus (SFV) [J].J Biotechnol.2019,10;304:63-69.

[13] Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China (Volume III)[S].Beijing: China Medical Science Press,2020:155.

[14] Cleaveland S, Kaare M, Knobel D, et al. Canine vaccination--providing broader benefits for disease control [J]. Vet Microbiol. 2006, 5;117(1):43-50.

[15] Rusche B.The 3Rs and animal welfare - conflict or the way forward? [J].ALTEX. 2003;20(Suppl 1):63-76.

[16] Löwa A,Jevtić M,Gorreja F,et al.Alternatives to animal testing in basic and preclinical research of atopic dermatitis[J].Exp Dermatol. 2018 May;27(5):476-483.

[17] Kawahara M,Takayama-Ito M,Kato H,et al.Development of an assay for detecting the residual viable virus in inactivated rabies vaccine by enzyme-linked immunosorbent assay[J].Biologicals.2021 Apr;70:59-63.