Replacement Study on the Potency Test of Anti- rabies Immunoglobulin in China

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ABSTRACT

Objective: To validate the relativity between the rapid fluorescent focus inhibition tests (RFFIT) and mouse neutralization test (MNT), In order to replace potency test of anti-rabies serum/ immunoglobulin. **Method:** The sample and reference standard were diluted 3-fold serially in 96-well microplate, then mix with a certain amount of challenge virus causing infection of 80%~95% of cells. Incubated at 37 for 1hr for neutralization in vitro, then adding BSR cell to incubate for 24hr and subjected to fluorescence staining. The results were observed by fluorescent microscopy. The 5th national standard of anti-rabies immunoglobulin was exam with RFFIT and MNT method by the 5th international standard of anti-rabies immunoglobulin. Validate the repeatability of two methods. Detect the samples of sera, equine rabies immunoglobulin and human rabies immunoglobulin with two methods to validate correlation. **Result:** the GMT potency of 5th national standard of anti-rabies immunoglobulin was 23.8IU/ml (RFFIT) and 21.4IU/ml (MNT), and the coefficients of variation were 13.3% and 62.3%. Detect 48 samples with RFFIT and MNT, the results show that the correlation coefficient of the two methods was 0.98, and has a close correlation. **Conclusion:** RFFIT method can be successfully detected the potency of anti-rabies immunoglobulin, and it can be the alternative method of MNT.

Abbreviations: NIBSC: National Institute for Biological Standards and Control, NIFDC: National Institute for Food and Drug Control, FITC:fluorescein isothiocyanate.

Introduction

Rabies is a fatal acute infectious disease caused by the crazy dog was infected with the rabies virus after injury. Human and animal rabies were preventable but cannot therapy. the effective treatment method of internationally recognized for post-exposure patient was wounds effective and correct cleaning process, then treated with antiserum or immune globulin, the third step was vaccine immunization. According the WHO guidance, the patient will determine the effective protection as long as the vaccine neutralizing antibodies

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produced by the body to achieve ≥ 0.5 IU/ml [1, 2]. Determination of the neutralizing antibody that neutralized in mice brain was established in the 1960s. The methods that neutralized in mice brain has been used for all kinds of various serological results of clinical trials of the vaccine validation. It's also known as MNT method.

However, from the mid-1980s, due to the MNT method poor repeatability or precision, and the need of large number of animals which does not comply with international experiments animal 3R. Furthermore, experimental time of the animal in vivo method requires a minimum of 14 days, so WHO and many countries drug agency suggest the method of rapid and reproducible in vitro, that is namely the rapid fluorescent focus inhibition test (RFFIT) and fluorescent antibody virus neutralization test (FAVN) [3,4,5].

Materials and Methods

1. Viruses and cells: CVS IP10 and the BSR cells were both from the Pasteur Institute in France, and adapt to the passage and amplification to establish three seed lots, take the working seed lot to CVS-9 for RFFIT detection. The BSR cells were passaged with DMEM media and establish two cell banks which stored in liquid nitrogen. The 5th International standard of anti-rabies immunoglobulin was from NIBSC, and antibody standards for the the five batches national anti-rabies virus antibody standard. Fluorescence (FITC)-labeled anti-rabies virus nucleoprotein monoclonal antibody was from Chemicom.

2. Sample to be tested: the 5th national standard of anti-rabies immunoglobulin was from NIFDC, Clinical serum which vaccination with rabies purified vaccine (Chiron Behring Vaccines Private Ltd) and the anti-rabies immunoglobulin were from NIFDC.

3. Reagents and instruments: Evans Blue, 96-well cell culture plate, DMEM, trypsin, gentamicin, fetal bovine serum, PBS buffer. Inverted fluorescence microscope (Olympus).

4. Operating procedures of RFFIT: Add 100μl dilution buffer into the wells of a 96-well cell culture plate, then add 50μl sample and standards into the wells for 3-fold series dilution. Neutralizing for 1hr at37 ° C after adding 50μl pre-titrated CVS virus each hole, while set the virus back-titrated group and cell control group. After neutralization add 50μl BSR cells (1 x 10^6/ml) each well, then cultured for 24 hours in 5% CO_2 incubator at 37 °C. Then washed the cell by PBS buffer and fixed

for 30min at 4 ℃ by 80% pre-cooled acetone. Discard the acetone and add 50μl FITC-labeled anti-rabies virus nucleoprotein monoclonal antibody, and incubated for 30min at 37 ℃. Washed three times by PBS buffer, adding 2 drops of 80% glycerol each hole, and observe fluorescent focus by the inverted fluorescence microscope. Records the fluorescent rate per well which in 50% of the upper and lower two dilutions, Calculate the potency through the reed-much method (reed muench).

5. Operating procedures of MNT: To dilute the serum samples and the standard by two-fold, using the PBS buffer which containing 2% fetal bovine. Add a certain amount of challenge virus (CVS strain), then incubated for 1hr at 37 ° C water bath. Take mouse of 10-12g, and brain cavity injection with each 0.03ml. Observed for 14 days, according to the incidence of the number of dead mice, calculated the potency of samples by Reed Muench method.

Detailed operating reference to the Appendix XIJ in the People's Republic of China Pharmacopoeia Volume III(2010 edition) .

6. Repeatability: Detect the potency of the candidate national reference anti-rabies immunoglobulin by the RFFIT method and MNT methods respectively, and the test standard is the 5th international standard of anti-rabies immunoglobulin. Repeat detecting for 25 times.

7. Correlation: Detect the potency of 48 kinds of the anti-rabies serum/immuno-globulin sample by the RFFIT method and MNT methods respectively, and the test standard is the 5th national standard of anti-rabies immunoglobulin. The potency result was been statistical analysis with paired rank sum test and scattergram analysis by SPSS11.5 software.

Results

1. the fluorescent focus criteria of RFFIT: after the neutralization of the test serum and the standard with a certain amount of virus, and inoculated BSR cells for 24hr.Check the virus by fluorescence staining, if the virus is fully neutralized there'll be no fluorescence, on the contrary, different degrees fluorescent focus can reflects the amount of virus which NOT be neutralized [6]. The fluorescent staining and fluorescent focus mode under the microscope shown in Figure 1. Record the highest serum dilution of 50% focus upper, and the

serum dilution of 50% focus down, The potency of serum to be test was calculate with the standard which potency is known.

2. The repeatability validation of the RFFIT and MNT methods: The candidate national standard of anti-rabies immune globulin was test 25 times, and the calibration results are as follows: the geometric mean (GMT) result of MNT method was 21.4IU/ml. The geometric mean (GMT) result of the RFFIT was 23.8IU/ml. The coefficient of variation (CV %) of the two detection methods were 62.3% and 13.3% respectively by statistical analysis. The above results showed the good repeatability of the RFFIT.

3. The correlation of the RFFIT and MNT methods: Total of 48 batches anti-rabies immuneglobulin/serum were test by the RFFIT and MNT methods, and the potency results are shown in Table 1. The paired rank sum test of the data from table 1 indicated that no difference between the two detection methods because the P = 0.059 (> 0.05, no statistically significant). The scattergram analysis of RFFIT and MNT using the data of table 1 (see Figure 2) showed that the correlation coefficient of the two methods is R=0.98(p <0.001, with significant statistical), significance. The above results indicate that there is a very good positive correlation between RFFIT and MNT methods.

Table 1 Potency of the anti-rabies serum/ immunoglobulin product by RFFIT and MNT

Sample	RFFIT (IU/ml)	MNT (IU/ml)
H1	107.2	131.0
H2	108.1	134.0
H3	52.5	68.0
H4	58.8	77.1
H5	80.8	73.6
H6	58.6	70.0
H7	64.2	75.9
H8	94.3	89.2
H9	36.5	37.2
H10	27.8	25.4
H11	27.8	25.5
H12	114.0	138.0
H13	48.5	69.0
H14	51.5	74.0
H15	146.0	132.0
E1	383.0	537.0
E2	231.3	195.2
E3	158.2	172
A1	4.8	1.2
A2	4.1	0.7
A3	3.7	4.2
A4	4.9	17.0
A5	5.2	1.3
A6	7.1	4.2
A7	6.5	1.7
A8	5.4	1.3
A9	12.4	13.5
A10	4.9	0.9
A11	5.4	1.3
A12	19.5	17.0
A13	8.8	1.9
A14	4.9	1.2
B1	2.4	2.1
B2	3.7	1.3
B3	2.8	2.6
B4	1.0	0.8
B5	2.8	0.8
B6	2.9	1.3
B7	7.9	2.6
B8	3.8	0.8
B9	11.9	21.4
B10	7.96	3.81
B11	6.7	6.6
B12	7.1	2.6
B13	23.9	30.2
B14	5.4	1.3
B15	3.7	1.3

3. The correlation of the RFFIT and MNT methods: Total of 48 batches anti-rabies immuneglobulin/serum were test by the RFFIT and MNT methods, and the potency results are shown in Table 1. The paired rank sum test of the data from table 1 indicated that no difference between the two detection methods because the P = 0.059 (> 0.05, no statistically significant). The scattergram analysis of RFFIT and MNT using the data of table 1 (see Figure 2) showed that the correlation coefficient of

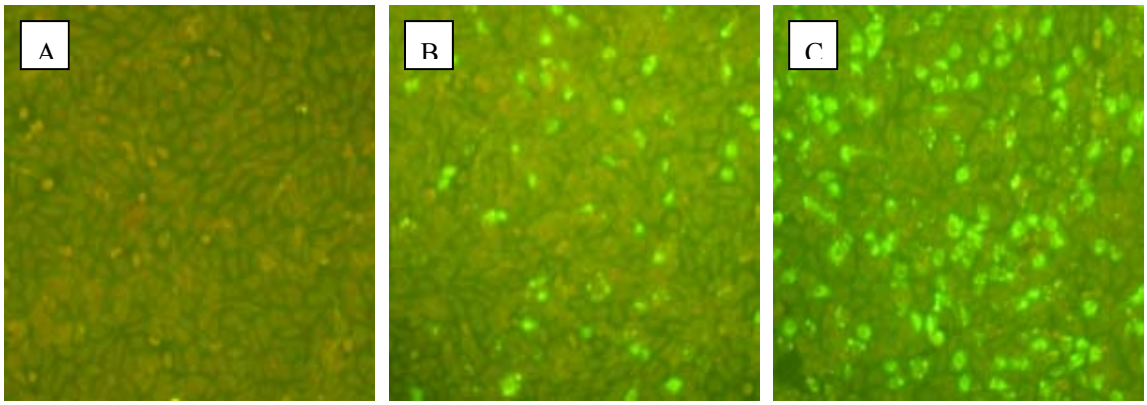

Figure 1: the fluorescent staining fluorescent focus mode of RFFIT

A: The negative cell control.
B: the highest dilution of serum that can inhibit 30% CVS virus.
C: the highest dilution of serum that can inhibit 85% CVS virus.

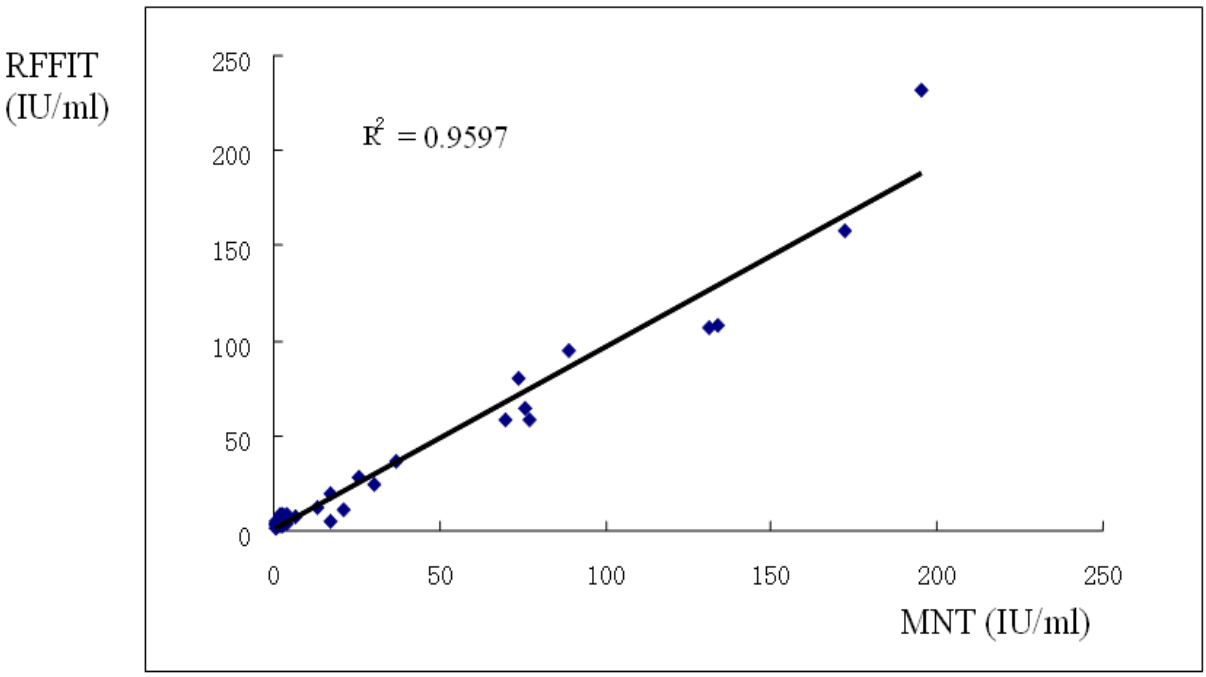

Figure 2: The scattergram analysis of RFFIT and MNT with 48 batch anti-serum/ immunoglobulin

the two methods is R=0.98($p < 0.001$, with significant statistical), significance. The above results indicate that there is a very good positive correlation between RFFIT and MNT methods.

Discussion

The basic principles of detecting anti-rabies virus neutralizing antibodies by RFFIT method was mixed challenge rabies virus and the serum to be test in vitro, and neutralized for 1hr. Add the BSR cells after the finishing of virus - serum mixture, and continue to inoculate 24hr. Stain the cells with FITC labeled anti-rabies virus nucleoprotein monoclonal antibody. If the virus was neutralized by the serum to be tested, the fluorescent focus would be negative or decrease, on the contrary, no neutralizing activity in the sample, the fluorescent focus would be positive. So the potency of the sample could be calibrated by the anti-rabies serum/immunoglobulin that having a known potency [8].

By RFFIT method, the rabies virus in cell culture period was only for 16-24 hours, so the progeny virus was controlled only in intracellular stage. In addition, the nucleoprotein of rabies virus in replication process is transcript firstly, then the nucleoprotein and the virus RNA formed to be the union rib-nucleoprotein that is stable, and the amount of it accounted for more than 36% of total protein. So the nucleoprotein was easy to be detected. That is to say the virus detected by RFFIT is the first generation of progeny virus that was not neutralized in cell culture. By MNT method, the virus that has not been neutralized in vitro was injected into the mice brain, and only the amount of the virus reaches a certain level enough to cause mice to death. Record the median lethal dose, then the potency of the sample could be calibrated by reed muench method with the anti-rabies serum/immunoglobulin. Various factors, such as the health of mice, breeding environment, injection techniques and so on, can both affect the result of MNT method. so the MNT method has poor repeatability [9-12].

Because the RFFIT method spend little time (only 2 days), and can detect a large number of samples once time, and do not require the use of animals (3R principles of experimental animals), So WHO strongly recommended the method, and

the method has been the approved by the agency of drug regulatory of China for the potency detection of anti-rabies serum/immunoglobulin now.

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