



Microtitration of Rubella Virus in Monovalent Vaccinal Products

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Abstract

Background: Potency test for control of rubella vaccine is a significant factor to qualify production line and vaccination program. For this reason, WHO recommends to use the microtitration method by both vaccine companies and control laboratories. Then the study was done to improve this test.

Methods: Three rubella virus samples, including an in-house standard, a lot of vaccine and an in-process product, were tittered in cell culture tubes. Then micro titration steps were tested on 96-well microplate using cocultivation of standard rubella vaccine dilutions and RK-13 cell line. After 6-7 days, final reading was done and calculated the titer. Two other samples were assayed with the micromethod.

Results: Titer reduction less than 0.5 log was acquired for each sample during frequent tests and between two methods.

Conclusion: The procedure was profitable and accurate for potency and identity tests of rubella virus vaccine, on the basis of WHO recommendations.

Keywords: *Microtitration, Rubella virus, Potency test, Microplate*

Introduction

Rubella virus is an agent of congenital rubella syndrome (CRS) with severe clinical complications like neurologic deficit, heart disease and eye manifestations (1). Otherwise its postnatal infection is usually mild and subclinical but it can be associated with joint symptoms, thyroiditis and encephalopathy (2). Nevertheless it is a preventable disease and a live attenuated rubella vaccine can induce a strong and enduring immune response (3, 4). Therefore effective potency of the vaccine is a significant factor in successful immunization. Viral titration, on the basis of cytopathic effects observation on cell culture is done in flask, tube and plate (5, 6), although titration in microplate is faster and more precise method for potency and identity tests of monovalent rubella vaccine (7). This project was conducted to study and to use 96-well microplate in titration to assay viral content of rubella vaccine, according to WHO manuals (8).

Materials and Methods

At first stage, in Human Viral Vaccines Department of Razi Institute, Karaj, Iran (in 2005), a batch of monovalent live attenuated rubella vaccine was selected as sample vaccine. In addition, lots of intermediate products were separated as in-process (harvest) sample. These two samples were used simultaneously with an in-house standard rubella vaccine to assay potency of virus. Then standard vaccine was diluted with DMEM⁺ medium (including tris buffer), in 10^{0.5} steps, from 10⁻¹ to 10^{-4.5}. Since rubella virus is light sensitive, it should be protected from direct light during testing. Then confluent Rk-13 cell culture tubes were washed with 1-1.2 ml of phosphate buffer saline (PBS). After then prepared dilutions were inoculated on characterized tubes (0.1 ml/tube). The tubes were incubated for 1 h at room temperature. Finally they received DMEM⁺ supplemented 0.5 µg/ml of antibiotic mixture (ka-

namycine and neomycine) and 1% calf serum (CS), as maintenance medium (1-1.2 ml/tube). Then they were incubated at 37° C. After 3 d, emergence of rubella virus CPE was studied with inverted microscope (Fig. 1). Afterwards their media were changed. On 7th day, final reading of CPE was done and titer of standard vaccine was calculated using the karber formula. The test was repeated 10 times for in-house standard vaccine. Moreover, two other samples, vaccine and harvest, were assayed with the tube method (macromethod). In the second stage, to optimize titration on flat bottomed microtiter plate in our laboratory, growth medium was prepared from DMEM⁺ supplemented with 8% CS and 0.3% antibiotic mixture. Two compounds were tested as maintenance medium. The first one included DMEM⁺+1% CS +0.3% antibiotic mixture. The second one contained 2% CS instead of 1%. Then a rang of cell concentrations in 10⁴ steps (1.1~2×10⁵ cell/ml) were prepared with trypsinization and cell counting of a flask of Rk-13 cell line. Then 100λ/well of every cell suspensions was cultured on 6 wells. From a kind of maintenance medium (100λ /well) were added to two separated wells of each concentration. Paper of clear label was adhered on wells and microplate was covered with lid. Then it was incubated at 32-33 °C in 5% CO₂. After 2-3 days, a confluent monolayer was provided using concentration of 1.6×10⁵ cell/ml and maintenance medium containing 2% CS and 0.3% antibiotic. For inoculum, viral dilution was examined on three volumes: 50λ, 75λ & 100λ per well, separately. Of course because of using maintenance medium, total volume per well was fixed as 200λ. After checking of cell for cytopathic changes, positive wells were observed in microplate of 50λ inoculum. After 6-7 d, final reading of CPE was done and titer of in-house standard vaccine was obtained. This method was tested nine other times for standard vaccine. Then it was repeated 10 times for both vaccine and in-process samples. At the third stage, following preparation of a 10³ TCID 50/ml suspension of standard vaccine, rubella antiserum was inactivated at 56 °C for 30 min-

utes. Then it was diluted in two-fold range. In that manner seroneutralization test was done to titer rubella antiserum on microplate (5, 9).

Moreover, rubella antiserum was made with dilutions as 1/100, 1/200 & 1/300. The dilutions and undiluted antiserum were used to detect a best dilution, without cell toxicity effect with rubella neutralization ability. This test was repeated two other times. In next process, viral suspension 10³ TCID 50/ml was provided from vaccine and in-process samples. For identity test, both late suspensions were neutralized one by one with rubella antiserum dilution of 1/100.

Results

In the first stage titration of rubella virus in standard vaccine was done 10 times on tube of cell culture (Table 1). Then results of potency tests were gathered for sample of vaccine and in-process, on the basis of macro method: 10^{4.7} TCID 50/ml and 10^{4.9} TCID 50/ml, respectively. In next stage, addition of 2% CS to maintenance medium was resulted to form a confluent monolayer at 32-33 °C in CO₂ incubator. Among the used range of cell suspension, 100λ per well of concentration 1.6×10⁵ cell /ml showed a complete monolayer during 2-3 day in 5% CO₂. Also proper viral inoculum per well was 50 λ to cocultivate with cell suspension and to create CPE. Thus in potency test of rubella virus was added 50 λ of viral dilution, 50 λ of maintenance medium and 100 λ of cell suspension per well. In other words total volume was 200 λ. This method was performed 10 times for in-house standard, rubella vaccine and harvest sample of rubella (Table 2). Following 3 times microtitration of polyclonal rubella antiserum determined mean of its titer: 1/256 .On the other hand, proper titer of antiserum was 1/100 to neutralize virus (10³ TCID 50 /ml), in identity test.

Table 1: Titer of in-house standard in tube method

Test No.	1	2	3	4	5	6	7	8	9	10	Titer Mean
Log Titer	4.5	5.0	5.0	4.5	4.75	4.75	4.5	5.0	4.75	4.75	4.75

Titer: $10^{4.75}$ TCID₅₀ /ml

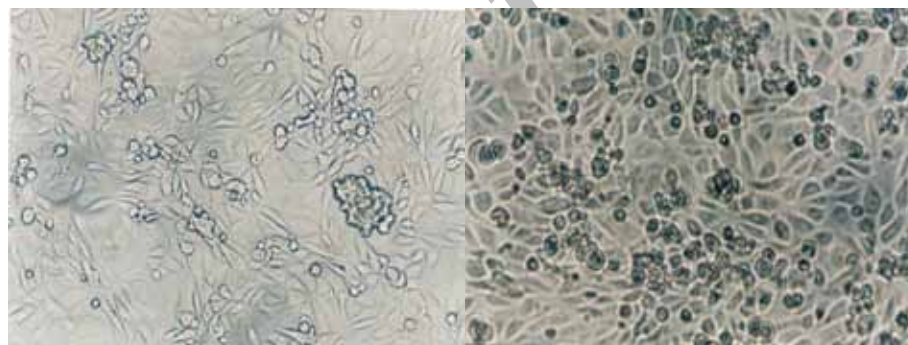
Table 2: Titer of samples with microtitration

Test No.	1	2	3	4	5	6	7	8	9	10	Titer Mean
In-house standard	4.5	4.6	4.6	4.6	4.9	4.9	4.5	4.5	4.5	4.5	4.6
Log Titer	4.3	4.4	4.3	4.3	4.2	4.5	4.5	4.5	4.4	4.5	4.4
Sample vaccine	4.4	4.6	4.4	4.4	4.5	4.5	4.5	4.8	4.4	4.5	4.5
Harvest sample											

Titer of in-house standard vaccine: $10^{4.6}$ TCID₅₀ /ml; Titer of Sample vaccine: $10^{4.4}$ TCID₅₀ /ml; Titer of Harvest sample: $10^{4.5}$ TCID₅₀ /ml

Table 3: Reduction of titer log between macro and microtitration

Titer of rubella virus	Standard vaccine	Sample vaccine	Harvest sample
Titer log in tube	4.75	4.7	4.9
Titer log in plate	4.6	4.4	4.5
Log difference	0.15	0.3	0.4

**Fig. 1:** RK-13 cell line (left: 16X magnification); Rubella CPE on RK-13 cell monolayer (right: 40X magnification).

Discussion

The main goal of rubella vaccine production is prevention of CRS with vaccination of 15-25 yr old women (10, 11). Titration of viral particles is an important test for control of final product before vaccine release, in accordance with standard manuals of WHO and vaccine companies. Growth of live attenuated virus of vaccine on cell culture and CPE observation is a golden and reliable test to assay titer of vaccinal strain (9, 12). Using 96-well micro plate to co-culture rubella vaccine and RK-13 cell line is a micro method for economic and rapid potency test (8, 9). In this project, maintenance medium with 1% CS (used in tube of macro method) did not form a confluent

monolayer of RK-13 cell line, contrary to medium with 2% CS, during 2-3 d. In addition, comparison of different cell suspensions showed the lower cell concentrations than 1.6×10^5 cell/ml was not able to create complete monolayer and upper than it was accompanied with overgrowth. Then in high concentrations, CPE observation was difficult (12). Moreover viral dilution inoculums of 75λ and 100 λ, against 50λ prevented continuous following up slow appearance of rubella virus CPE (9). However, it is usually recommended 50 λ inoculum and number of cell, i.e. $1-2 \times 10^5$ cell/ml that should be optimized for each laboratory (8, 9, 12, 13). Also micro titration of the rubella antiserum showed that to prevent

cytotoxicity, it should be diluted. Microscopic appearance of the toxicity was different from rubella CPE.

According to WHO standard, if results discrepancy during frequent titrations were ≤ 1 log, reported conclusions were accepted (8). In our investigation, titer differences in the consecutive micro method tests were 0.5 log about each of samples, separately (Table 2). In addition, result of difference between two methods was <0.5 log for the samples, one by one (Table 3).

Thus, micro method of rubella virus on microplate could be a good replacement for macro method in tube. It is noticeable the micro titration is an economic, fast and exact procedure to assay rubella particles in monovalent vaccine (7, 12). As a result it should be diluted and used in identity test.

Ethical Considerations

Ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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