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Respiratory Syncytial Virus Isolation and Titration

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Abstract:- *Measuring the virus titer is one of the most important procedures in virology, is the concentration of viruses in a sample. End point assay is a widely used approach for determining the quantity of infectious virus preparation. Respiratory syncytial virus was isolated from clinical samples. The concentration of the viral preparation was determined by this methods, TCID50, the result obtained from the first method was 1×10^7 virus/ml which is equal to 7.2×10^6PFU/ml*
Key words: *Respiratory, virus, concentration, assay.*

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I. Introduction

Virus quantification involves counting the number of viruses in a volume to determine the virus concentration. It is important in both research and development (R&D) in commercial and academic laboratories as well as production situations where the quantity of virus at various steps is variable. For example, in production of viral vaccines, recombinant proteins using viral vectors and viral antigens all require virus quantification in order to continually adapt and monitor the process to optimize production yields and respond to all changing demands and applications.These methods are into two categories, traditional and modern methods. Traditional methods are industry-standard methods which have been used for decades, they are slow and labor-intensive. Modern methods are new commercially available products and kits that reduce quantification time (1).

Human respiratory syncytial virus (RSV) is a virus that causes respiratory tract infections. It is a major cause of lower respiratory tract infections and hospital visits during infancy and childhood. In temperate climates there is an annual epidemic during the winter months. In tropical climates, infection is most common during the rainy season . Nearly all children will have been infected with the virus by 2–3 years of age (2).Some of those infected with RSV, 2–3% will develop bronchiolitis, necessitating hospitalization. Natural infection with RSV induces protective immunity which wanes over time—possibly more so than other respiratory viral infections—and thus people can be infected multiple times. (3). RSVinduced bronchiolitis results in the hospitalization of >100,000 infants per year in the US. RSV is also a significant cause of respiratory illness in high-risk adults and the elderly (4). Despite the importance of RSV as a respiratory pathogen, the pathogenesis of primary RSV infection is not fully understood, and effective vaccines and efficacious post-infection therapies are not currently available Risk factors for severe RSV disease include age less than 1 year, age less than 3 months at the start of the RSV season, bronchopulmonary dysplasia, congenital heart disease,and prematurity (5). Not surprisingly, immunodeficient and immunosuppressed children and adults are highly susceptible to RSV infection. In a retrospective study of 111 bone marrow transplant recipients with documented pulmonary infection, RSV was the most commonly detected pathogen (6).

Maturation of the immune system in the first years of life is important for susceptibility to RSV disease. Immunological immaturity of infants limits antibody (Ab) responses to RSV infection and limits the immunogenicity of vaccines. Infants less than 8 months old produced lower levels of serum and nasal wash anti-RSV IgG and IgA than infants 9–21 months old. In a phase 1 trial of an RSV vaccine candidate, infants less than 5 months old did not produce serum neutralizing antibodies (nAbs) whereas children 6–24 months old responded with good nAbs (7). Maternal Abs present in infants likely confer partial protection from RSV infection, but maternal Abs may also suppress Ab and T cell responses to primary RSV infection ,Not only do infants produce less Ab to RSV than older children, but also anti-RSV Abs produced by infants are often undetectable 1 year post-infection, suggesting that Abs produced by infants are not as stable as Abs produced by immunologically mature individuals (8).

Human respiratory syncytial virus (hRSV) is an enveloped, single stranded, negative senseRNAvirus belonging to the genus Pneumovirus and familyParamyxoviridae (9). It is a medically important paediatric virus responsible for acute respiratory illnesses worldwide (3). The virus particle is composed of a helical

nucleocapsid enclosed by a lipid envelope studded with three transmembrane glycoproteins, G, F and SH (10). While the function of SH protein is yet unknown, the G and F glycoproteins mediate virus attachment and fusion, respectively, and are major targets of the protective host immune response (11).

II. Materials And Methods

3.4 Cell culture and manipulations:

The experiments were done following the protocols stated in the manual of technique and applications book of IianFreshney -6thed, 2010.

2.3.4.1 Passaging (Splitting/ Subculturing) Cells in Monolayer Cultures:

The trypsin/EDTA (25%) was defrosted in a 37^{o}C water bath and its temperature was left to come up to 37^{o}C. Cell medium was decanted. PBS was used to wash the cell layer. The trypsin/EDTA was added. The mix was swirled over the cells and places the flask in the 37^{o}C incubator for 10 minutes. Growth medium was added and swirled over the cells to mix thoroughly. Cells were spliced equally between fresh flasks and incubated at 37^{o}C.

2.2.4.2 Counting the cells:

A volume of 0.1 ml cells was added to 0.9 ml growth media in a separate tube. A small drop of diluted cell suspension was taken up into a pipette and a single small droplet was placed carefully onto the centre of the haemocytometer. The cover slip was placed over the cells; care was taken not to get any bubbles. The number of cells was counted under the microscope in each of the four sets of 16 squares. The total number of cells in these 64 squares was divided by 4 to obtain an average count of cells per 16 squares. Since the area is 1mm^2 and thedepth 0.1 mm

2.3.4.5 Isolation and Preparation of RSV Stocks:

The positive samples for hRSV were clarified by centrifugation at 1000 rpm for 30 minutes at 4˚C. A confluent T25 cm^2 monolayer of Hep2or A549cells was trypsinised andsplitted. Cell suspention (5 ml) was added into fresh flasks in concentration of 1×10^5 cell/ml. Growth medium was added (10ml) per flask and flasks were incubated at 37°C and 5% CO_2 in the humidified CO_2 incubator for 18 hours. The growth medium was removed from the flasks by inversion into a sterile beaker. The cell monolayer was washed with 10 ml PBS. Viralinoculums in volume of 0.5 ml per flask were added and the flasks wereshacked gently to ensure that the inoculums reach all parts of the monolayer. Maintenance medium added.

The flasks were incubated at 37ºC with CO2 for 2 hours (with continuous rolling every 10 minutes). The media was removed;the flask washed with PBS twice.Maintenance media 10 ml was added to each flask.The flasks were incubated at 33°C in the humidified CO_2 incubator. In the next day the lid of the flask of infected cells was opened to relieve the air pressure and closed again. Theincubation was continued. Syncytia were observed after 4 days of incubation. When large syncytia were observed and the monolayer began to detach after 8-10 days, the monolayer was scraped into the medium with a sterile rubber policeman. The flask's content was transferred into 50 ml conical centrifuge tube. The suspension was sonicated for 5 minutes at room temperature, and then it was centrifuged for10 minutes at 10000 rpm at 4°C. The supernatant was collected as the new virus stock; it was liquated in a volume of 2 ml in cryotubes and stored at -80°C.

Propagation of RSV in Vero Cell Line:

1.A confluent T75 cm^2 monolayer of Vero cells was trypsinised and splitted. Growth media was added in volume of 25 ml. The cell suspension was added to new flasks in concentration of 1×10^5 cell/ml into new T75 cm^2 flasks, growth medium was added to 25 ml final volume. The flasks were incubated at 37°C and 5% CO_2 in the humidified CO_2 incubator for 18 hours. One of the flasks was the control. The growth media was removed. The cell monolayer was washed with 10 ml PBS twice. Viral inoculums were added in volume of 0.5 ml to each flask except the flask of control. Maintenance medium was added and the flasks were incubatedat 33°C and 5% CO_2 in the humidified CO_2. In the next day the lid of the flask of infected cells was opened to relieve the air pressure and closed again. Theincubation was continued. Syncytia were observed in cell monolayer after 4 days of incubation. When large syncytia were observed and the monolayer began to detach after (9-10) days, the monolayer was scraped into the medium with a sterile rubber policeman. The flask's content was transferred into a sterile 50 ml conical centrifuge tube. The suspension was sonicated for 5 minutes at room temperature, and then it was centrifuged for10 minutes at 1000 rpm at 4°C. The supernatant was collected, liquated into the 2 ml cryotubes.

TCID 50:

Plates of 96 wells were seeded with A549, Hep 2 cells (5000cells/well) in growth media (100µl) and were incubated at 37°C in the humidified CO_2 incubator for 18 hours. The growth media was removed from the cell monolayers,washed with PBS and replacedwith 100µl of maintenance media in each well. In a tube 1ml of virus stock was added to 10ml of maintenance media. In a separate U bottom 96 wells plate, virus dilutions were done, a volume of 300µl was added to the first well and decimal dilutions of RSV virus were prepared by adding 30µl from the first well to 270µl of maintenance in the second well and mixed well by pipette before going to the next well. A volume of 100µl was taken from each well using multichannel pipette and was added to the first column of monolayer's plate.

3. From each dilution, 100µl was transferred to the plates to make a total of 200µl per well. The last column was left without virus as control. The plates were incubated at 37ºC with CO2 for 2 hours. The media was removed;the wells were washed with PBS twice. Maintenance media (100µl) was added to each well, the plates were incubated at 33°C in the humidified CO_2 incubator for more than 5 days. The media was removed;the wells were washed with PBS twice. Methylene blue stain (100µl) was added to each well, the plates were incubated33°C in the humidified CO_2 incubator for 1 hour. The plates were washed under tap water.
Using the method of Reed & Muench (11).

III. Results And Disuccion

Virus stock was prepared after (8-12) days of incubation at 33ºC from infected monolayer when the monolayer detached from the base of the flask.The resuls showed that TCID50 of RSV stocks was2×10^8TCID50/ml which is equal to1.38×10^8 PFUs/ml. This endpoint dilution assay quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells. The figure (3-4) represents one of the plates that used to calculate TCID50 value.

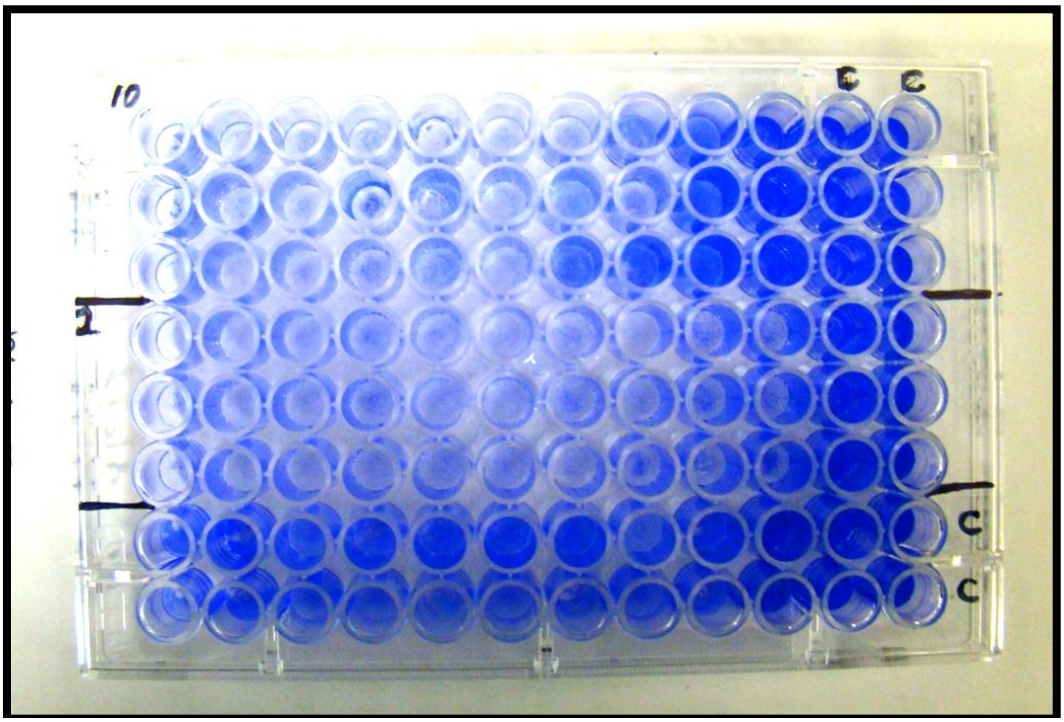

Figure (3-4): TCID50of RSV virus in (A549) cell line monolayer.The first three rows represent 10 fold RSV dilutions; the next three rows represent 2 fold RSV dilutions. The last two rows and columns represent mock cells. The light blue represents the dead cell while dark blue represent live cells after infection with RSV.

This study is comparable to many studies done by authers like Flint *et al*. in 2009 (12)that this method takes more than a week. Also many studies reported that this method can take up to a week due to cell infectivity time (13).

In our study calculation of TCID50 is important to detect the virus concentration in order to be used in the other experiments involved infection with RSV. Many studies revealed that virus quantification is utilized in both research and development in commercial and academic laboratories as well as production situations, where

the quantity of virus at various steps is an important variable. For example, the production of viral vaccines, recombinant proteins using viral vectors and viral antigens all require virus quantification to continually adapt and monitor the process in order to optimize production yields and respond to ever changing demands and applications (14).

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