

Multiplicity of infection and incubation period for fixed rabies virus strains propagation on BHK-21 and Vero cell lines

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Abstract

Introduction: Rabies is a viral zoonotic disease under the genus *Lyssa* virus, family *Rhabdoviridae* which can affect all warm-blooded mammals including humans. The disease is 100% fatal if post exposure prophylaxis is not in place with effective vaccine timely. Production of quality cell culture vaccine is required for effective control and prevention of the disease. To produce high quality and effective vaccine, setting viral multiplicity of infection and incubation period at a pilot step is required.

Objective: To determining effective multiplicity of infection and incubation period for fixed rabies virus strains propagation on Baby Hamster Kidney-21 and Vero cell lines.

Materials and Methods: Vero and Baby Hamster Kidney-21 cell lines were infected with Eveninyl rokipinik avelset and Pastuer virus fixed rabies virus strains at 0.1, 0.01 and 0.001 tissue culture infectivity dose per cell and incubated for 48, 72 and 96 hours at 37 °C. Viral cultures were harvested and determined for its titer and compared across tissue culture infectivity dose and incubation period.

Results: According to growth kinetics of cell lines, Vero cell line takes more than 72 hours to reach confluence, where as BHK-21 cell line reach confluence before 72 hours incubation. For Eveninyl rokipinik avelset virus on Vero cell line, the result from titration indicate high virus titer ($10^{7.75}$ LD₅₀/ml) with the lowest tissue culture infectivity dose (0.001) per cell and high incubation period (96 hours); whereas Pastuer virus virus on Baby Hamster Kidney -21 show high titer ($10^{7.50}$ LD₅₀/ml) with highest tissue culture infectivity dose (0.01) per cell and lower incubation period (72 hours) compared to Vero cell line.

Conclusion: For best virus titer during virus culture, 0.001 tissue culture infectivity dose with 96 hours incubation period on Vero cell and 0.01 tissue culture infectivity dose with 72 hours incubation period on Baby Hamster Kidney -21 cell were selected for Eveninyl rokipinik avelset and Pastuer virus fixed virus strain respectively.

Key words: Cell culture, rabies virus strain, multiplicity of infection, incubation, virus titer

Introduction

Rabies is a viral disease that causes acute inflammation of central nervous system in both animals and humans. The disease is worldwide problem with more than 55,000 human deaths per year; majority occur in Asia and Africa due to socio-economic problems and limited access to effective anti-rabies vaccines available today (WHO 2007). The virus introduced through animal bite or scratch, and travel to brain in retrograde movement from the site of inoculation. Dog is the major domestic animal responsible for rabies transmission with 95% and other domestic and wild animals contribute the rest (Peter 2015). Once the sign of rabies develop, it's almost 100% fatal but the disease can be prevented by vaccination before onset of the disease.

Following introduction of the first anti-rabies vaccine by Pasteur in 1885, several anti-rabies vaccines were developed using different substrates for virus propagation (WHO 1999). World Health organization banned first generation of anti-rabies vaccines; Fermi type due to several reasons. The major problems

associated with these vaccines are low immunogenicity due to antigen content, and vaccination associated complications. Due to presence of high content of myelin of the adult brain tissue in nerve tissue based vaccine, probability to develop nerve paralysis after injection is the major problem associated with this vaccine. In addition, nerve tissue based vaccine is discouraged by the recipient due to high dosage (seventeen injections for full immunization) on umbilicus with 2-5 cc compared to only five injections (IM) in the case of modern cell culture based vaccine (Neuza 1998). At the same time, immunogenicity is very high in the case of cell culture based vaccine and protective antibody can be developed following the third injection (WHO 1996; Wiktor 1969).

The challenge of anti-rabies vaccine production in developing countries is associated with lack of modern technologies to change production system from nerve tissue to tissue culture (Peter 2015). Using cell cultivation for anti-rabies vaccine production can easily be scaled up and high virus titer

can be produced resulting high immunogenicity vaccine (Anandan 2014). Importantly, cell culture based anti-rabies vaccine production reduces the cost of mass production making affordable for low income countries without safety concerns (Montagnon 1989). Low virus titer in the production system affect final potency of the vaccine and need to determine the best effective tissue culture infectivity dose (TCID) at which maximum virus titer can be reached. This can be supported with antigen concentration technique to get high antigen value for best immune response following vaccination (Paldurai 2014). Therefore, the study aimed to set effective TCID per cell and incubation period to produce Eveninyl rokipinik avelset (ERA) and Pastuer virus (PV) rabies virus with high titer using Vero and Baby Hamster Kidney (BHK)-21 cell lines to produce safe and efficacious anti-rabies vaccine.

Materials and Methods

Cell lines: Mammalian cell lines, BHK-21 (originated from primary hamster kidney) and Vero (from kidney of African green monkey) were grown in 75 cm² tissue culture flask (invitrogen) using Dulbecco minimum essential medium (DMEM) with 10% fetal calf serum. Confluent cells were split and counted to determine number of cells per milliliter of suspension.

Fixed rabies viruses: Cell culture adapted Pasteur virus (PV) and Eveninyl Rokitinki Abelset (ERA) fixed rabies virus strains were obtained from Center for Disease Control (CDC-Atlanta) and titrated to know tissue culture infectivity dose per milliliter of virus suspension before use. The results of seed virus infectivity titer were used to set multiplicity of infection and to calculate virus to cell ratio during virus infection.

Virus production: Depending on seed virus titer, virus to cell multiplicity of infection (TCID) set as 0.1, 0.01 and 0.001 viruses per cell. At the same time, incubation periods were selected as 48, 72 and 96 hours with the above different TCID for each incubation periods. Confluent cell lines were split and counted to know cell density per milliliter. Based on cell count, 5 x 10⁶ cells infected with different virus multiplicity of infection per cell (0.1, 0.01 and 0.001) for both BHK-21 and Vero cell lines. After incubation at 37 °C for 30 minutes to allow virus penetration to cell lines, infected cell suspension transferred to 25 cm² tissue culture flasks containing complete medium, and incubated at different incubation periods as stated above.

Titration: The supernatant from virus cultures were collected and stored at -80°C. Samples were taken

from all virus cultures and titers were determined according to the principle of in vitro virus titration using 96 well micro-titration plate. Ten fold dilution of the virus sample prepared and distributed on 96 well micro-titration plates, each dilution contain minimum four replicas. After incubation with cell lines for 48 hours at 37 °C in the presence of 5% CO₂, plates were washed and stained with anti-rabies fluorescent isothiocyanate conjugate (FITC). Reading was performed using fluorescent microscope and virus titer calculated according to spear-man kerber formula.

Results

Cell and virus growth kinetics: After virus infection at different TCID, cell growth was followed daily for cell surface confluence using inverted microscope. During incubation period for each TCID, healthy cell growths were recorded and then transferred to -80 °C at the end of stated incubation period. Media pH change due to cell and virus growth was maintained by adding sodium bicarbonate at each follow up time. After 72 hours following freezing, virus suspension were collected and sampled for further virus titration.

Virus titer: Using standard virus titration method, obtained virus harvests were titrated and different virus yield were found at each infection rate and incubation period. Virus titers were also varying between BHK-21 and Vero cell lines even at the same incubation period and multiplicity of infection. Similarly, the two virus strains showed different virus yield at similar incubation period and multiplicity of infection.

Table 1: Virus titration result (ERA virus on Vero (LD₅₀/ml))

Multiplicity (TCID/cell)	Virus titer (TCID ₅₀ /ml)		
	48 hrs	72 hours	96 hours
0.1	10 ^{5.75}	10 ^{5.50}	10 ^{4.75}
0.01	10 ^{4.75}	10 ^{6.00}	10 ^{6.25}
0.001	10 ^{4.5}	10 ^{5.75}	10 ^{7.75}

Table 2: Virus titration result (ERA virus on BHK-21(LD₅₀/ml))

Multiplicity (TCID/cell)	Virus titer (TCID ₅₀ /ml)		
	48 hours	72 hours	96 hours
0.1	10 ^{4.50}	10 ^{5.75}	10 ^{5.50}
0.01	10 ^{5.00}	10 ^{5.25}	10 ^{6.00}
0.001	10 ^{5.75}	10 ^{6.50}	10 ^{6.25}

Table 3: Virus titration result (PV virus on Vero (LD₅₀/ml))

Multiplicity (TCID/cell)	Virus titer (TCID ₅₀ /ml)		
	48 hours	72 hours	96 hours
0.1	10 ^{5.75}	10 ^{5.50}	10 ^{5.25}
0.01	10 ^{5.25}	10 ^{5.75}	10 ^{6.50}
0.001	10 ^{4.75}	10 ^{6.25}	10 ^{6.75}

Table 4. Virus titration result (PV virus on BHK-2(LD₅₀/ml))

Multiplic y (TCID/cell)	Virus titer (TCID ₅₀ /ml)		
	48 hours	72 hours	96 hours
0.1	10 ^{4.25}	10 ^{5.50}	10 ^{5.25}
0.01	10 ^{6.25}	10 ^{7.50}	10 ^{6.75}

0.001	$10^{5.75}$	$10^{6.25}$	$10^{6.50}$
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The nature of Vero cell line growth is slower compared to BHK-21 taking more than 72 hours to reach full confluence. The longer incubation period expected to achieve maximum virus titer with the lowest TCID (Table 1). Virus penetration to cell line is slower and takes time to reach maximum titer, thus require longer incubation period. This indicates the nature of Vero cell lines slow growing kinetics with 96 hours incubation periods to reach maximum cell confluence (Table 2 and 4).

From the growth kinetics, BHK-21 cell lines were observed to be the most replicating and fast growing with 72 hours maximum incubation period to reach confluence. The titer of the virus is affected by cell confluence nature during cell replication and thus different infection rate show variable result based on incubation period and TCID (Table 2 and 4). At the same time, as the virus replicate using growing cells, the pick at which high virus titer with confluent cell lines is important to get maximum effective virus titer.

Discussion

Before the discovery of cell culture in 1950's, animals like mice, rats, rabbits and embryonated eggs were used for virus propagation (Mohammed et al. 2015). As rabies is one of the deadly diseases but can be prevented by vaccination, producing safe and efficacious vaccine is the major effort required to control the disease. The effort requires mass vaccination of reservoir animals, mostly dogs; safe and efficacious vaccine production at large scale is required which can be achieved by modern cell culture technique. To produce effective cell culture anti-rabies vaccine, careful selection of cell to virus infection ratio with appropriate incubation period is mandatory before starting mass production (Webster and Clow 1936). For best virus titer, different infection ratio (0.1, 0.01 and 0.001) virus to cell ratio were used as study points with variable incubation period (48, 72 and 96 hours) for both ERA and PV fixed rabies virus strains. From virus to cell growth ratio, as the virus require cells to replicate, the rate at which cell lines replicate directly affect replication of the virus and the virus also affect growth of cell lines. At a given pick point, virus to cell growth ratio reach maximum and it can be taken as best titer for further mass production (Maria et al. 1988). In this study, it is observed that BHK-21 cell line is fast growing and can reach maximum confluence within 72 hours, and start to detach from growth surface indicating that further incubation strongly affect the titer of the virus as the cell line dies (Leslie and Anna 1937). In other case, Vero cell lines show longer incubation period

without detaching from the surface, thus longer incubation period can increase virus titer as the virus still replicating in growing cells, similar to work done by Mohammed et al. 2015.

Virus titration result similar with cell growth kinetics indicating maximum virus titer for Vero cell line at longer incubation period compared to BHK-21. Tissue culture infection ration is also one of the major growth rate determining factors indicating that as virus to cell infection ratio increase, cell growth should be at high rate with lower incubation period. At the same time, the lower incubation period for the cell lines to reach confluence, the higher virus to cell infection ratio used and vice versa. This can reflect lower virus to cell multiplicity with high incubation period allow virus to infect cell lines slowly without affecting cell replication and can reach maximum virus titer at 96 hours incubation period. Accordingly, ERA fixed virus strain on Vero show high virus titer ($10^{7.75}$ LD₅₀/ml) with 0.001 viruses to cell infection ratio at 96 hours incubation period. In other, PV fixed virus strain on BHK-21 show high virus titer ($10^{7.50}$ LD₅₀/ml) at 72 hours incubation period with 0.01 viruses to cell infection ratio.

Conclusions

Based on this study, it can be concluded that the best virus titer obtained at 96 hours incubation period with 0.001 ratio and at 72 hours incubation period with 0.01 infection ratios for ERA and PV fixed virus strains respectively. Thus, for mass vaccine production, it is recommended to use 0.001 infection rates with 96 hours incubation period for ERA virus on Vero cell lines and 0.01 infection rates with 72 hours incubation for PV virus on BHK-21 cell lines.

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