BHK-21 Cell Grown on Microcarrier System Increasing the Capacity of Rabies Vaccine

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***Abstract*—Rabies is a fatal zoonotic disease caused by the rabies virus. Vaccination is the most effective way to prevent and control rabies. Pusvetma as one of national rabies vaccine producer needs to increase the production capacity to meet the market demand of this vaccine. The use of microcarrier for propagating the Baby Hamster Kidney (BHK)-21 is expected to increase production capacity of Pusvetma's rabies vaccine. This study aims to compare the efficacy of two propagation methods of BHK-21 cell: 1) microcarrier system using bioreactor and 2) conventional method using roller bottle. Microcarrier cell culture using 2 × 10^8 cells in bioreactor contain 850 carriers, whilst conventional method using 26 roller bottles contain 5-10 × 10^6 cells/bottle. We propagated 65 ml of Rabies virus Pasteur strain into microcarrier cell culture and 5 ml same virus into roller bottle (virus titer 10^7 $LD_{50/ml}$). Our result showed that BHK-21 cell growth was 12.96 × 10^8 cells with 500 ml Eagle Medium, virus titer $10^{6,7}$ $LD_{50/ml}$ in bioreactor, and 13 × 10^8 cells with 5200 ml Eagle medium, virus titer obtained was $10^{6,0}$ $LD_{50/ml}$ in conventional method . The concluded was the usage of microcarrier more effective and efficient than conventional method.**

Keywords—BHK-21 cell, microcarrier, rabies virus, vaccine.

I. INTRODUCTION

Rabies is zoonotic disease caused by Rabies virus that belong to genus Lyssavirus and family Rhabdoviridae. Taxonomical classification of Lyssavirus is based on antigenic differences of nuckleoprotein (N). There are 6 genotypes and rabies virus is belong to genotype 1 [1]. Rabies is estimated to cause 59.000 human deaths annually in over 150 countries with 95% of cases occuring in Africa and Asia. Due to widespread underreporting and uncertain estimates, it is likely that this number is a gross underestimate of the true burden of disease. The main reservoir and vector of rabies disease are dog and rabies event on human is 99% caused by dog's bite [2]. Rabies is a vaccine-preventable disease. Vaccinating dogs is the most cost-effective strategy for preventing rabies in people. Dog vaccination reduces deaths attributable to rabies and the need for post-exposure prophilaxis as a part of dog bite patient care [3]. Vaccination program is primary choice in controlling and elimination of rabies disease in Indonesia which supported by other related program to ensure if vaccination program is doing well, such as post vaccination survey, fast response to bite case, handling of vaccine cold chain and also directed elimination program which notice to animal welfare aspect, and good handling of veterinary drugs and coordination mechanism and report of the implementation of vaccination program [4]. Rabies vaccine has been known since 1879, first produced by Victor Galtier. Furthermore in 1880, the vaccine is developed by Louis Pasteur who made rabies vaccine from virus which originate from infected-rabies canine bone marrow and passaged in rabbit brain and then attenuated. In 1958 Kissling cultured CVS rabies virus in cell culture of baby hamster kidney. Moreover in 1963 Kissling and Reese succeeded to make inactive rabies vaccine by using rabies virus which cultured in baby hamster kidney cell (BHK) [5]. The terminology of cell is used first by Robbert Hooke (1635-1703) to express the basic unit of organism which could propagate [6]. In the first, microcarrier system is developed to propagate suspended cell, that could be easily applicated in stirred bioreactor tank, could be measured and the effectivity shall be validated conveniently. Microcarrier cell system has many advantages compared to conventional system which use bottle, that is could reduce the amount of bottle and could enhance cell density. Microcarrier cell system has been used for polio vaccine and rabies vaccine industries [7]. Pusvetma as a producer of rabies vaccine in Indonesia need to improve the method to increase production capacity. The preliminary research is held as method development to enhance production capacity of rabies vaccine in pusvetma by compare BHK-21 cell propagation in *microcarrier* using bioreactor with conventional system using roller bottle.

II. MATERIALS AND METHODS

A. Propagation of Rabies virus using the microcarrier system

Four roller bottles of BHK-21 cell is washed using by PBS solution, added with 5 ml Versen-Trypsin and homogenized. Furthermore, homogenized with 20 ml cell-contained Eagle`s medium using pipette until the whole

cells are separated each other, followed by cell counting using hemocytometer.

120 ml Eagle medium with 10% Bovine Serum contain 2×10^8 BHK-21 cells and 0.918×10^8 BHK-21cells inoculated into two *CelCradle*™-500 (bottle I and bottle II) which contain 850 carriers, shake it gently for every 15 minutes at the first 1 hour and every 30 minutes at next 3 hours and then measure glucose concentration and medium pH. After more than 90% BHK-21 cells adhere on carriers, then add medium up to 500 ml. Set the bottles in *CelCradle*™ that put in a 5% CO_2 incubator at 37 °C.

Parameter *CelCradle*™

Up rate	: 1 mm/sec
Top holding time	: 10 sec
Down rate	: 1 mm/sec
Bottom holding time	: 30 sec

BHK-21 cells attached to the microcarrier were counted by using the haemocytometer. Monitoring of medium pH and glucose are carried out every day and if glucose concentration less than 100 mg/dL, then add glucose and L-glutamine[8].

65 ml Rabies virus strain Pasteur (virus titer 10^7 $LD_{50/ml}$) inoculated into BHK-21 cell of cradle bottle that contains 12.96×10^8 BHK-21 cell (bottle I) and 7.072×10^8 BHK-21 cell (bottle II) , incubated at temperature 33 °C for 1 hour and added virus medium growth up to 500 ml, incubated for 4 days and then harvested rabies virus suspension. Measurement of rabies virus titer using Spearman-Kärber method, the minimum of expected virus titer is 10^7 $LD_{50/ml.}$

Parameter *CelCradle*™

Up rate	: 1 mm/sec
Top holding time	: 30 sec
Down rate	: 1 mm/sec
Bottom holding time	: 10 sec

B. Propagation of Rabies Virus in BHK-21 Cell using monolayer system.

Propagation of BHK-21 cell from roux bottle to roller bottle, set up 26 roller bottles with total of $5\text{-}10 \times 10^6$ cells per roller bottle and placed 72 hours in incubator at 37 °C. The same Rabies virus seed is inoculated with dose 5 ml per roller bottle which contain $0.5\text{-}1.0 \times 10^8$ BHK-21 cell incubated at temperature 33 °C for 1 hour and added 200 ml virus medium growth, incubate for 4 days and then harvest the virus. Measurement of Rabies virus titer using Spearman-Kärber method [9]. The minimum of expected virus titer is 10^6 $LD_{50/ml}$.

C. Rabies Virus Titer Measurement

The extracellular virus titer from cradle bottle and roller bottle were determinted through test carried out in mice weight 18 – 20 gram by using Spearman-Kärber method [9]. The dilution 10^{-1} until 10^{-6} of the virus suspension were intracerebrally inoculated with 0.03 ml/mice. The animals were observed for 14 days. The minimum of expected virus titer is 10^6 $LD_{50/ml}$.

III. RESULTS

The initial cell density for inoculation were 235.934 and 108.000 per carrier, respectively. The cell density of the BHK-21 cells grown on microcarriers reached 1.600.000 and 862.400 per carier, respectively, following 7 days of inoculation. There has been an increase in cell density up to 580% and 698%.

Cell BHK-21 growth on microcarrier using two *CelCradle*™ bottle were presented at Table 1 and Table 2.

TABLE 1. BHK-21 CELL GROWTH MONITORING AT CELCRADLETM IN BOTTLE I

Day	pH	Glucose mg/dL	Amount of Cell	Sample	Carrier	Amount of Cell per Carrier
0	7.3	345	2×10^8	0	850	235.294
1	7.0	271	-			
2	7.0	255	0.65×10^8	10	850	76.470
3	7.0	170	1.65×10^8	10	840	196.428
4	7.1	213	3.00×10^8	10	830	361.445
5	6.7	132	4.58×10^8	10	820	558.536
7	6.4	78	12.96×10^8		810	1.600.000
	7.0	364	12.96×10^8		810	1.600.000

TABLE 2. BHK-21 CELL GROWTH MONITORING AT CELCRADLETM IN BOTTLE II

Day	pH	Glucose mg/dL	Amount of Cell	Sample	Carrier	Amount of Cell per Carrier
0	6.7	147	0.918×10^8	0	850	108.000
2	6.7	-	1.316×10^8	10	850	154.800
4	6.8	181	2.646×10^8	10	840	315.000
5	6.7	87	3.257×10^8	10	830	392.400
7	6.4	48	7.072×10^8	10	820	862.400

Growth of BHK-21 cells in the monolayer system with the initial $5\text{-}10 \times 10^6$ cells per roller bottle has become $0.5\text{-}1.0 \times 10^8$ BHK-21 cell after 4 days incubation.

Cell BHK-21 growth on microcarrier using roller bottle were presented at Table 3.

TABLE 3. BHK-21 CELL GROWTH MONITORING MONOLAYER SYSTEM

Day	pH	Glucose mg/dL	Amount of Cell per Roller Bottle	Amount of Cell in 26 Roller Bottle
0	7,2	330	5×10^6	130×10^6
4	6.8	255	0.5×10^8	13×10^8

Rabies virus titer are presented in Table 4.

TABLE 4. RABIES VIRUS TITER

Sample*	Mycrocarrier System	Monolayer System
Harvest - H1	$< 10^{6.0}$	-
Harvest - H2	$10^{6.0}$ $LD_{50/ml}$	-
Harvest - H3	$10^{6.4}$ $LD_{50/ml}$	-
Harvest - H4	$10^{6.7}$ $LD_{50/ml}$	$10^{6.0}$ $LD_{50/ml}$

*H1= virus harvested on day 1
H2= virus harvested on day 2
H3= virus harvested on day 3
H4= virus harvested on day 4

IV. DISCUSSION

Roux flask, roller bottle or bioreactor are method which often used for production of cultured cell products. The utilization of microcarrier using Bioreactor could increase cell density [8]. According to [6] there are 3 cell growth phases, to wit: lag phase (adaptation phase), in this phase, cells still adapt to its growth medium so that the cells become flat relatively, logarithmic phase (exponential phase), cells have well adapted to its growth environment so that the cells have doubling time shorter than prior phase, stationary phase, the amount of lived cells is equal with death cells because of nutrient concentration decreases, and if it is carried on would come into death phase.

The result of cell propagation until day 7 is 12.96×10^8 cells per cradle bottle. That result is equal to monolayer BHK-21 cell in 26 roller bottle with total of cells approximately 0.5×10^8 cells per bottle. According to [9], bioreactor system is designed based on flux and reflux movement principle which compression and decompression aspiration might do intermittent exposure between nutrition and air to cells. This makes shifting lower, high aeration and free-foamed culture microenvironment so those maximize nutrition absorption and oxygen transfer. Because of efficient nutrition and oxygen transfer, one *CelCradle*™ bottle could produces cells which equal with 18 to 20 roller bottles. The usage of microcarrier provides larger surface area approximately 15.000 cm^2 and this could make cell growth more than 10^{10} cells in 4 bottles with size 8.6" × 11.7".

Efficiency of BHK-21 cell propagation with microcarrier system is the amount of used eagle medium. The volume of eagle medium is 500 ml with one-time medium exchange for 500 ml compared to 26 bottles × 200 ml is 5200 ml medium. This is only need one-time cell propagation compared to 13 times of splitting from roux bottle to obtain 26 roller bottles. There are some other efficiencies, to wit: materials saving, time saving of equipment preparation which begin from washing process, sterilization, medium filtration and serum, and time to provide equipment and its process, and also it could be utilized for placing employee at another production process.

Glucose concentration and pH could affect cell growth significantly [10]. Control of pH and glucose concentration are always monitored by pH meter dan GluCell because nutrient of Eagle medium will decrease as cell metabolism increase. If medium pH is more acid then should be added natrium bicarbonas and if glucose concentration is under 100 mg/dl then should be added glucose and glutamine in order to make appropriate growth medium and cell propagation could occur.

The result of virus titer from harvested *CelCradle*™ sample day 4 is $10^{6,7}$ $LD_{50/ml.}$ While virus titer from roller bottle sample is $10^{6,0}$ $LD_{50/ml.}$ This might be because of limitation of oxygen transfer which emerge in adsorption step and affect cell metabolism and further cell development [11].

V. CONCLUSION

BHK-21 cell growth on microcarrier system using bioreactor has produced high density cells it reduces usage of roller bottles, medium and reagents compared with conventional system using roller bottle could provide more efficient system to produce rabies vaccine in large scale.

VI. SUGGESTION

To complete graphic of BHK-21 cell propagation in microcarrier, it still needs repetition of this system with constantly monitoring of pH and glucose concentration in order to obtain higher rabies virus titer so that it could support increasing of production capacity of rabies vaccine of Pusvetma.

VII. LIMITATION

The Mini bioreactor microcarrier system is first step to develop to bioreactor tank system which expected to provide virus suspension in larger production scale. In this research, there are not all of the equipment possessed by Pusvetma, to wit: CelCradle and GluCell.

ACKNOWLEDGMENT

The authors would like to thank Nandini Prabakhar, Ph.D. (Bioprocess Scientist) Esco Aster CDMO Services, Singapore. This work was supported by Laboratory of Rabies Vaccine Production, Pusat Veteriner Farma, Surabaya, Indonesia.

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