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Standardization and Assessment of Cell Culture Media Quantities in Roller Poly Ethylene Terephthalate Bottles Employed in the Industrial Rabies Viral Vaccine Production

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Abstract: Vero cells are utilized for production of rabies vaccine. This study deals with the optimize quantity media require for the rabies vaccine production in the smooth roller surface. The rabies virus (Pasteur vaccine strain) is infected to monolayer of the various experimented bottles. To analyze the optimal quantity of media for the production of rabies viral harvest during the process of Vero cell derived rabies vaccine. The trials are started from 200 to 400 mL (PTARV-1, PTARV-2, PTARV-3, PTARV-4 and PTARV-5). The samples are taken in an appropriate time intervals for analysis of In Process Quality Control (IPQC) tests. The collected viral harvests are further processed to rabies vaccine in a pilot level and in addition to scale up an industrial level. Based on the evaluation the PTARV-2 (250 mL) show highly encouraging results for the Vero cell derived rabies vaccine production.

Key words: Rabies virus, Vero cells, roller bottle, rabies vaccine

INTRODUCTION

Rabies is a worldwide fatal disease and represents a severe public health problem in developing countries. Domestic animals, mainly dogs and cats, or wild animals (bats, monkeys and foxes) are important virus reservoirs and transmit this disease to human and animals, such as cattles, horses and others. The dogs are major vector for rabies transmission throught the world and are responsible for 94% of the estimated 60,000 deaths from rabies per year (Martinzn, 2000). Post-exposure treatment and immunization of the professionals at risk (pre-exposure scheme) in this country approximately 2,500,000 vaccine doses are used per year (Neuza *et al*., 2004). After the discovery by Pasteur a century ago, rabies vaccines have been produced with rabies virus obtained from the brain of experimentally inoculated animals. Unfortunately, they are not always very immunogenic and free of untoward reactions. Furthermore, it is difficult, using animal brains as a source of virus, to achieve large scale production. The introduction of the animal cell culture technology in the field of rabies vaccine production about twenty-five years ago has opened up a new era. Preparation of purified, concentrated, potent and well-tolerated rabies vaccines has become feasible, for both medical and veterinary use. The production of viral vaccine is more time consuming than those compared with the bacterial vaccines several cell culture systems have been proposed for mass production of anchorage-dependent cells and viruses for fundamental studies. Nowadays, rabies cell-culture vaccines are produced on different cell lines derived from mammals or apes. Diploid cell lines, such as MRC-5 cells (a human fibroblast cell line) or heteroploid cells like Vero cells (WHO, 2007) (a continuous cell line of African Green monkey kidney epithelial). The cell line Vero has been recommended by the World Health Organization for the production of human vaccines. For the large scale production of vaccines culture conditions should be optimized to support high density growth of Vero cells. Human rabies vaccine produced on Vero cells is a good alternative to the human diploid cell vaccine. Likewise, Vero cells has been largely used to manufacture other viral vaccines, such as polio (Montagnon *et al*., 1984), influenza (Kistner *et al*., 1998) and Japanese encephalitis vaccine (Sugawara *et al*., 2002). The most widely used system is the roller culture bottle but more efficient methods have been proposed with varying degrees of success viz., the spiral plastic film, the perfusion system, glass beads, rotary column and multi surface propagator. Microcarrier has been proposed and seems promising. Most of these systems are commercially available, but none has gained overall acceptance (Corbell *et al*., 1979). The introduction of the micro-carriers and bioreactors technology has recently made possible the production of these new vaccines in

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an industrial scale. It must be noted, nevertheless, that such modern technologies require sophisticated and costly equipment as well as high-level trained personnel and that it may be difficult to transfer these technologies to the developing countries, which are eager to use them in order to increase and improve their vaccine production. The World Health Organization is paying much attention and devoting much effort in trying to help resolving this situation. For an economical and efficient process, providing the best environmental conditions for cell growth and product formation must optimize productivity. While some mammalian cells grow in suspension culture, most are anchorage dependent cells, which must attach to a suitable substrate surface before they become physiologically functional. The cell surface Physical and chemical interactions between the cell, the substrate to which it will attach and the suspending (i.e., growth) medium as well as the cells' production of adhesions will influence the number of cells which will attach and their strength of attachment. Mammalian cells have been shown to attach to numerous substrates including glass, a variety of polymers and even metal. Polystyrene, a negatively charged, hydrophobic polymer, is commonly used but, untreated it is a poor substrate for cell attachment (Whiteside and Spier, 1987; Hendrick *et al*., 2001). Treating a polystyrene surface with proteins such as polylysine or fibronectin makes it more hydrophilic with an overall positive charge favoring cell attachment. Treatment with chromic or sulphuric acid increases the overall negative charge resulting in an increase in adsorption of serum proteins and this favors cell attachment (Ramsay *et al*., 1997). The effect of ammonia on the growth of hybridoma cells has been examined in both, batch and continuous cultures. Static batch cultures were carried out with initial ammonia-concentrations in the medium. Lactate might inhibit cell growth in systems like fed-batch, where due to extension of the culture period, the cumulative amount of consumed glucose and subsequently the amount of produced lactate increases (Otzturk, 2006). A summary of possible mechanism for the inhibitory effect can be found by Godia and Cairo (2006). This study deals with the optimization and evaluation of the roller bottle culture for the production of the Vero cell derived rabies viral harvest. A safe and potent vaccines produced by cell culture techniques are available in developed countries and are used in humans for pre-and post exposure prophylaxis.

MATERIALS AND METHODS

Vero cells: Vero cells (CCL-81) were obtained from the American Type Culture Collection (ATCC) at passage number 125 as monolayer's and as frozen cells. Master Cell Bank (MCB) were prepared at 130th passage level. The MCB was stored in a vapour phase of a liquid-nitrogen container. From the MCB the Vero cells are further expanded as Manufacturers Working Cell Bank (MWCB) were established at 136th passage level. Biosafety tests of the cell banks were performed as per requirement and successfully passed.

Revival of cells: Stored vials containing Vero cells were taken from liquid nitrogen and immediately placed in a sterile beaker containing sterile distilled water (DH_2O) at 37°C for rapid thawing. Cells were transferred quickly into two roux bottles containing growth medium (100 mL each) with 10% serum. The cells were incubated at 37°C to form a monolayer. To remove toxic DMSO and dead cells, a medium change was done after 12-15 h.

Media used for cell culture: Eagle's Minimum Essential Medium (MEM) with non-essential amino acids, phenol red and 1.5 g L^{-1} of sodium bicarbonate, pH 7.0 to 7.5 (Hi-media, Bombay) was used for Vero cell culture. The dry powder was hydrated with triple distilled water to 1X concentration. For maintenance of Vero cells, the MEM with 2.2 g L^{-1} of Sodium bicarbonate instead of 1.5 g L^{-1}. used to maintain the pH range of 7.5±7.6. For pH adjustment CO_2 was used. Required quantities of Neomycin and New Born Calf Serum (NBCS) were added at the time of batch culturing.

Roller culture

Smooth surface disposable roller bottle: The Disposable Polyethylene Terephthalate (PET) roller bottles with smooth surface, sterile, flexible and break proof were used (Greiner bio-one, gmbh). The bottles used were designed (DNAase/RNAase free) as non-pyrogenic. It measures about 116×276Ø (mm)×height (mm). The maximum volume of the roller bottle was 2500 mL provided with growth area of 850 cm^2.

Propagation of Vero cells: Growth medium from the bottles showing confluent monolayer were removed and washed with warm PBS to remove dead cells, serum component and to enhance the trypsin activity. Small quantities of 0.25% trypsin in Dulbecco's saline A with sodium bicarbonate was added to each bottle and incubated at 37°C for few minutes for the detachment of cells. Based on cell count, cells were dispersed in growth medium and transferred to roux/roller bottles, incubated at 37°C for 48 h.

Cell counting: Five milliliter of Vero cell culture were washed three times with Phosphate Buffered Saline (PBS)

and treated with 5.0 mL citric acid (Sigma) containing 0.1% TritonX-100 (Merck USA) and incubated at least for 1 h at 37°C. The released nuclei were counted using heamocytometer (HBG company).

The specific growth rate, μ (h-1) was calculated by the following equation.

$$U = L_nX_n - [L_nX_{n-1}]$$

where, X represents the variable cell density per mL, t the time points of sampling expressed in hours and the subscripts n and n-1 stand for succeeding sampling points.

Cultural analysis of Vero in smooth surface roller bottle: The vero cells are experimented in various levels of media i.e., 200, 250, 300, 350 and 400 mL and are marked PTARV-1, PTARV-2, PTARV-3, PTARV-4 and PTARV-5 (Pasteur Tissue culture Anti Rabies Vaccine). These bottles are taken to the analysis different culture parameters like passage, pH, RPM, virus titer.

Analysis of cell yield and pH: In each experimental bottles with initial inoculum of 19 million cells mL^{-1} were analyzed for their cell yield in subsequent passages (141, 142 and 143). Samples are collected upto 14 days from the experimental bottles to analyze the pH. All the experimental bottles incubated in various rpm's conditions like 0.3, 0.6 and 1.2. The initial cell inoculum is 19 million cells mL^{-1}. attachment of the cells, confluency reaching is assessed daily.

Virus: Fixed strain of Pasteur virus (PV-11) obtained from Institute Pasteur France was initially adopted to primary cultures of fetal bovine kidney cells and later adopted to Vero cells. The mother seed (PV-RAGE-01) was stored in liquid nitrogen. Master virus seed bank was prepared from the mother seed on Vero cells by harvesting the viral fluid, stabilized with 10% New Born Calf Serum (NBCS) and distributed in 5 mL quantity into 50 mL vial and stored at -80°C/Liquid nitrogen. Working virus seed bank was prepared from master virus seed bank as described above and tests for sterility, titer and adventitious agent were carried out.

Virus propagation: Roller bottles were examined under microscope, washed with calcium and magnesium free PBS, infected with virus seed material having a virus titer of 10^{-5}(10 mL per roller bottle) and incubated at 35°C for about 30 min for adsorption of the virus. Then about 300 mL of maintenance medium was added per bottle. For maximum yield of virus from the cell culture, the exact Multiplication of Infection (MOI) relating to the infective virus material and the number of viable cells in the culture were analyzed and used. Five percent of the bottles were kept as control culture bottles for the extraneous viral agents' detection by Heamadsorption test.

Titration of Virus infectivity: The virus infectivity in mice (LD_{50}) was done as per the technique described by Koprowski (1973). Groups of 10 mice were inoculated intra cerebrally with 10 fold dilution of the viral harvest, each mouse receiving 0.03 mL. The mice were observed 14 days and recorded the mice mortality from 5th day onwards from the date of inoculation. Any mice showing signs of fixed-virus rabies infection (paralysis, convulsions) between 5th to 14th days were included and the titer was calculated by the method of Reed and Muench (1938).

Viral harvests: The supernatant fluids containing virus material were harvested into large sterile bottles and the same bottles containing infected cells were used further for another 4-5 harvests by replenishing with maintenance medium at an interval of 72 h. The rabies virus comes out in the fluid by budding out process and it does not produce any cytopathic effect (CPE) in the infected cells.

Concentration, inactivation and purification: Based on titer value, viral harvests were pooled and filtered through pre filter 0.45 μm membrane and collected, as Single Viral Harvest (SVH). The SVH were further concentrated by ultra filtration in a Tangential Flow ultra Filtration (TFF) system (Millipore JMBM 0137) (Nathan *et al*., 2008) with a molecular weight cut off 100, 000 polysulfone membrane (BIOMAX) was used for concentration. The SVH was concentrated to 20X level by volume. Both retentate and filtrate materials were subjected to mouse titration test to check the integrity of the system and the run. After the concentration the material was diafiltered with required volume of Phosphate Buffered Saline (PBS) pH 7.2. After checking the virus titer the concentrate was further subjected to inactivation with β-propiolactone (1:3000). The inactivation was ensured by *in vivo* as well as *in vitro* method and by estimating the residual βPL content. The inactivated concentrate was subjected to purification by affinity chromatography (Matrex Cellufine sulphate, Amicon B.NO 412950, Japan) method. Samples were taken during the process for In Process Quality Control (IPQC) tests like adventitious agents, antigen titration in BHK-21 cell line and identity. The other tests carried out were potency assay, protein concentration, detection of residual cellular DNA. The potency of the vaccine was set at 6.0 IU/dose for verifying of viral inactivation, potency, protein concentration, detection of residual cellular DNA.

Vaccine preparation: The purified materials with potency of 6 IU (International Units) per milliliter were used to prepare the vaccine. After appropriate dilution of stabilizers like human albumin and maltose were added to have a final concentration of 1 and 5%, respectively. The vaccine batches were packed singly. Samples of these vials were used for potency, adventious agents, safety, stability and other efficacy tests.

RESULTS AND DISCUSSION

The PTARV-2 bottle attained confluency on fourth day in 143rd passage when compared with other bottles. Among the five trials, high cell concentration was obtained from the PTARV-2 and early confluency was achieved at 142nd and 143rd passage when compared with 141st passage. Strength of the pH were maintained between 7.1-7.4 in PTARV-2 it was normal when compare with the others. The pH of the other trails were fluctuating between neutral to alkali and to acidic (Table 1). The optimization of rpm for culturing and propagation reveals that the confluency reached on 4th day at 6 rpm in PTARV-2, whereas in other trial volumes and different rpm the cells took more than 6 days to reach confluency in spite of the increased media volumes (Table 2). The viral titre are higher in during the propagation of rabies virus, the viral titre of Viral Harvest was 2.6 that titre are higher when compare other experimented bottles harvests. And also the titre entire harvests are to some extent higher. Table 3 shows Rabies viral protein yield analysis in roller bottles different volumes of media in vero cell line during the industrial rabies viral vaccine production.

Table 1: Surveillance of pH during the culturing of Vero cells (passage of 143rd) in different trails volumes of media

	Days						
Volume (mL)	2	4	6	8	10	12	14
PTARV-1	7.2	7.4	7.5	7.8	7.4	7.4	7.5
PTARV-2	7.4	7.2	7.3	7.2	7.4	6.8	7.1
PTARV-3	6.9	7.4	7.6	7.8	7.9	7.8	7.9
PTARV-4	7.3	7.5	7.8	7.4	7.4	7.3	6.9
PTARV-5	7.1	6.9	7.4	7.3	7.3	7.8	7.6

In order to culture anchorage dependent cells in vitro is essential to know now the cells interact with their environment. A 'coat' called the glycocalyx surrounds the cell membrane. The glycocalyx consist of glycolipid, glycoprotein and transmembrane proteoglycans. A large number of these molecules contain sugars with a negative chare. So, the net charge on the glycocalyx is negative. That means that if a culture surface is positively charged there will be an electrostatic attraction between the cell and the culture surface (Wisseman and Jacobson, 1985). In PTARV-2 bottle there was a gradient decrease in the time taken to reach confluence on the subsequent passages. This result deliberates that the provided environment influences the cells to propagate faster and the cells got adapted to the provided niche (Wisseman and Jacobson, 1985). During culturing and passaging, media volume plays a vital role. Amongst the series of experimental media volumes, PTARV-2 of media yields 58 million of cells in the 141st passage and 63 million cells in 143rd passage this is the highest cellular yield when compared with other trial media volumes. On the other side, the pH values of the all trails are started with 6.9 to 7.4. At the end of the experiments (on the 14th day) the range were 6.9 to 7.6. The starting pH of the PTARV-2 was 7.4. It is a slightly high range. But the end of the experiment the pH was maintained as physiological range (7.1). The other experiments ph range are not in physiological range, it's an alkali as well as acidic conditions, the intracellular pH are decreased because of the ammonia generates an acidic conditions. It seemed that the pH has a significant influence on the effect of ammonia. Therefore, Ludemann *et al.* (1994) endorsed inhibition by ammonia to the undissociated NH_3 where concentration changes considerably with varying pH. According to their equations, the specific growth rate was reduced by 50% at NH_3 concentration of 0.095 mM^{-1}, corresponding to a total ammonia concentration of 5.6 mM^{-1} at pH 7.5. The variability in the pH of the trial volumes except PTARV-2 is due to the accumulation of unutilized proteins of serum and the accumulation of metabolites (Nahapetian *et al.*, 1986). Several waste

Table 2: Role of different RPM's in Vero cell (143rd) by different trail volumes of media

	Cell yield					
	RPM 0.3		RPM 0.6		RPM 1.2	
Volume	Cells in millions	Confluency reached on	Cells in millions	Confluency reached on	Cells in millions	Confluency reached on
PTARV-1	42	9th day	52	6th day	51	8th day
PTARV-2	41	7th day	58	4th day	49	9th day
PTARV-3	44	8th day	54	7th day	50	7th day
PTARV-4	46	7th day	50	5th day	45	6th day
PTARV-5	42	9th day	55	6th day	47	7th day

Table 3: Rabies viral protein yield analysis in roller bottles different volumes of media in Vero cell line during the industrial rabies viral vaccine production

S.No	Cell count	Qty of media used (mL)	VHI	VH2	VH3	VH4	VH5
PTARV-1	19×10^6	200	$10^{3.2}$	$10^{4.4}$	$10^{3.9}$	$10^{2.8}$	$10^{1.9}$
PTARV-2	19×10^6	250	$10^{4.3}$	$10^{5.5}$	$10^{4.9}$	$10^{4.1}$	$10^{2.6}$
PTARV-3	19×10^6	300	$10^{3.0}$	$10^{4.3}$	$10^{5.3}$	$10^{4.1}$	$10^{2.2}$
PTARV-4	19×10^6	350	$10^{2.8}$	$10^{4.3}$	$10^{4.1}$	$10^{3.5}$	$10^{1.9}$
PTARV-5	19×10^6	400	$10^{3.7}$	$10^{4.5}$	$10^{3.9}$	$10^{2.8}$	$10^{1.9}$

Common factors- MOI-0.1, Virus strain- PV11-VP 155, Virus titer-$10^{-5.3}$/0.03 mL. VH: Viral harvest, VP: Viral passage

products of cell metabolism have been reported to be inhibitory or toxic to cells. The most important substances mentioned in this context are ammonia and lactate. The accumulation of ammonia in the course of the cultivation leads to growth inhibition even when a process is kept under otherwise well controlled physiological conditions. At high ammonia concentrations, the quality of product can significantly decrease (Ryll *et al*., 1994). Lactate can be formed from sugars other than glucose and from glutamine. The toxic action of lactate is probably due to the effect on the pH and osmolarity of the culture medium, only occurring at relatively high concentrations (>20-30 mM) (Wentz and Schiiger, 1992). It seems ammonia and lactate formations are due to an inefficient overflow metabolism. In the presence of high concentrations of glucose and glutamine, glucose is rapidly metabolized by aerobic glycolysis and most metabolic energy is derived from glutamine oxidation. The accumulation of these metabolites to potentially inhibitory levels can to overcome by nutrient control by optimized level of media addition (Schneider *et al*., 1996). Inhibition by ammonia seems to play a much more important role. Thus, total concentrations of ammonia and ammonium as low as 2-3 mM has been reported to reduce cell growth considerably, depending on the cell line and culture conditions. Ammonia release by cells is due to amino acid metabolism, mainly that of glutamine. The latter is routinely added to many culture media since it has been shown to stimulate growth and antibody productivity (Flickinger *et al*., 1992).

Where large volumes of cells or culture fluid are required and the cells of choice are not adopted for spinner culture or require attachment to a substrate for growth, roller bottles offer the investigator a convenient though labor-intensive method for scaling up. A small roller rack can be placed in an incubator chamber, large incubator box, or hot room set to 35 or 37°C. Sterile disposable roller bottles are available. The virus yield experiment reveals that PTARV-2 mL of media yield high titer value beside the trial volumes. This is because the cells in the trail PTARV-2 utilize the proteins completely from the media and there is no accumulation of unutilized serum components and metabolite products. High density of cells was obtained in 6 rpm where it yields 58 million of cells in PTARV-2, whereas in all other rpm there was no high densities of cells were observed. Monolayers of Vero cells were infected with the rabies PV11 strain.

Multiple harvests can be obtained from the same roller bottle of cells by collecting the conditioned medium and adding fresh medium to the cells in the bottle. The second, third and later harvests most probably will be after a shorter culture time than the first, since the cells will have had time to grow to a higher density. The viral proteins are harvested from infected cultures from 3rd day after inoculation, five times of intervals of 72 h. The rabies virus titer was obtained by the intracerabrally mouse inoculation tests (Reed and Muench, 1938). During the rabies virus propagation in all the bottles (PTARV-1 to PTARV-5) the highest virus titer was obtained from PTARV-2 bottle. The other trails bottles viral titers are very low when compare with the PTARV-2 because of the higher concentration of ammonia and lactate. This results are same as well as in Hamadri and Bandyopadhyay (1994). The high concentration of ammonia and other metabolites ca result in the decrease of specific growth rate and final cell density the inhibition of virus proliferation on cells (Hamadri and Bandyopadhyay, 1994). In general the viral titer are decreased during consequent passages, but in the PTARV-2 viral titer of VH5 was higher it shows to get more doses. The all viral harvests of PTARV-2 (VH1 to VH5) are pooled and concentrated by pellicon cassette system (Millipore). Concentrated viral materials are inactivated with Betepropiolactone (βPL) of 1:3000 ratio (Peck *et al*., 1951). All the inactivated antigens are purified and formulated with human albumin and maltose. Prior releasing the formulated vaccines are inspected their standard by the guidelines of Indian pharmacopeia.

CONCLUSIONS

Production of tissue culture derived rabies vaccine is made from the optimal environmental conditions. Where in the environmental conditions would be fulfilled by various factors viz., temperature, pH, cell inoculums concentration, media protein concentration and adaptability of cell lines. In these experiments the other important factors like optimal quantity of media and the role of RPM during the culturing of cells, propagation of virus were analyzed. The concentrations of media are inversely proportional with cell adaption and yield. The cell density and viral titer are very low in media higher

concentration trails (PTARV-3 to PTARV-5). If the quantity of media decreased or increased, the culturing of cells to be fully deviated but these same factors is influenced during virus propagations. Virus propagation in the roller bottle with the surface area of 850 cm^2 and the trail PTARV-2 prove to yield virus of higher titer value when compared with increased volumes of media. The rabies highest titer value obtained by minimum quantity of cell culture media's to meet global needs. To attain higher cell concentration, viral titer with lower volume of media. To fulfill the global needs by low cost production of tissue culture derived rabies vaccine with utilized fewer amounts of media. Viral yield were increased in the trails PTARV-2 and it is optimized nutritional utilization of the cells. As the volume increase, the availability of the nutrition's also increases because cell surface is constant. This significant in increased accumulation of cellular toxicities like secondary metabolites which resulting due to the fluctuation range of pH (7.2 to 7.6) it directly impact of the lower yield of cell density as well as viral proteins

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