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Standardization of microcarrier based bioreactor culture in parallel with roller bottle for rabies virus (PV-11) propagation in Vero cell using MEM eagle's and RPMI 1640 medium

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Abstract

The Vero cell is the continuous cell line used as a cell substrate for many viral vaccines manufacturing including Rabies vaccine. The Pitman and PV-11 strains of rabies virus are commonly used for production of rabies vaccines. In our study the PV - 11 strain is used as seed virus for the production of Vero cell derived inactivated Anti Rabies Vaccines (Lyophilized). The working cell bank and working virus seed lot were prepared and tested for its sterility and mycoplasma contamination. In this study the roller bottle method and micro carrier (cytodex-1) based bioreactor culturing method were standardized with MEM Eagles and RPMI 1640 medium. The outcome of the two methods were compared ,to find out the medium and culturing method which can give higher concentration of Vero cells to get higher viral titre in mass production. The quantities of Vero cells obtained from passage 144 to 148 were assessed using a haemocytometer cell count procedure. The quantity of the Vero cell obtained per roller bottle ranged from 222.82x10^6 cells to 236.67x10^6 cells in MEM Eagles medium and 227.80x10^6 cells to 230.66x10^6 cells in RPMI 1640 medium. The average quantity of Vero cells in roller bottles were 1.12 x 10^6 cells per ml of culture. In the bioreactor culturing method the Vero cells obtained in the concentration of 1.69 to 1.75 million cells / ml of culture by MEM Eagles medium and 1.67 to 1.70 million cells / ml by RPMI 1640 medium. In the bioreactor the average yield of vero cells ranged 1.70 x10^6 cells per ml of culture. It is estimated that about 35% increase in Vero cell quantity in bioreactor system than the roller bottle culturing method. 0.3 MOI (Multiplicity of Infection) of rabies virus was kept as constant for infection of confluent monolayered Vero cells in both roller bottle and bioreactor culture. Four viral harvests were collected at three day intervals and replenished with fresh culturing medium each time. The viral titres in each viral harvest were quantified using in-house standardized RT-PCR method; the roller bottle method yielded viral titre log 6.044 to 7.106 and the bioreactor culture yielded log 6.559 to 7.216 rabies viral particles. It is observed that the bioreactor culture yielded more viral titre than the roller bottle culture using both the medium.

Keywords: Bioreactor; Rabies; Vaccine; Vero cell; MEM Eagle's; RPMI 1640; Cytodex – 1; Microcarrier; Virus titration

1. Introduction

Rabies is a highly fatal zoonotic viral disease and it can be preventable by timely intervention with effective vaccination. The "Semple"' rabies vaccine prepared from animal nervous tissue which was initially used, has been replaced with modern cell culture based vaccines preparation. The earlier nervous tissue derived rabies vaccine is potentially hazardous and has disadvantages of causing severe adverse reaction including neurological complications [1]. It was superseded in purity, potency and safety by the rabies vaccine prepared by cell culture technology and these vaccines are free from many side effects [2]. The Vero cell line *(Cercopithecus aethiops)* is a well known mammalian cell substrate

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system licensed by WHO for the manufacturing of vaccines like Japanese encephalitis, rotavirus, polio, yellow fever and rabies etc., [3, 4]. The mycoplasma contamination in the animal cell culture system is very common and prevention by filtration is very difficult because of its smaller size and lack of rigid cell wall. The starting material such as virus, cell substrate and serum requires effective method of screening for mycoplasma contamination. The Vero cell needs solid support for its attachment and growth. Various tools like cell culture flasks, Roux bottles, Roller bottles, cell cubes, cell factories and micro carriers are used for its anchorage support. In bioreactors, Cytodex - 1 micro-carrier beads are being used for the propagation of anchorage depended cells for the large scale manufacturing of vaccine [5]. The bioreactor culture reaches the higher cell density than the roller bottle and proportionately yields increased viral titre.

2. Material and methods

2.1. Cell Culture Media

Minimum Essential Medium (MEM) - Eagle's (Himedia Cat # AT047) and Roswell Park Memorial Institute Medium (RPMI - 1640 - Himedia Cat # AT028) were used in this study as cell culture media. The media were prepared by dissolving required quantity of dehydrated media in tissue culture grade water and sodium bicarbonate (Sigma S-5761) was used as pH stabilizer. The pH of the medium were adjusted to 7.2 by sparging sterile CO_2 gas using 0.22 µ cartridge filter. The Revival medium (10% FBS), Growth medium (5% FBS) and Maintenance medium (2% FBS) were prepared with respective percentage of Fetal Bovine serum (Gibco) with 1% Neomycin and 0.5% Amphotericin B.

2.2. Cell Line History

2.2.1. Master Cell Bank

The Vero cells are (African green monkey kidney cells - *Cercopithicus aethiops - ATCC)* used as host system for propagation of Rabies virus PV-11 strain. The master cell banks were preserved at vapour phase of the liquid nitrogen in the designated storage container and revived at passage level 140.

2.2.2. Working Cell Bank

The working cell bank was prepared by reviving the Vero cell in 25 cm^2 TC flask (passage 141), subcultured into 150 cm^2 TC flask (passage 142) and further subcultured into 850 cm^2 (passage 143) roller bottle using MEM Eagle's growth medium. The cells were resuspended in freezing media in the concentration of 10 million cells / ml. Each ml of the cell suspension aliquoted into cryovials, freezed by stratacooler followed by storage in liquid nitrogen vapour phase and used as working cell bank.

2.2.3. Rabies virus working seed lot

The confluent Vero cell monolayer in roller bottles (passage 145) were infected with 0.2 MOI of rabies virus PV-11 strain using virus stock containing 6.50 log of virus. Four viral harvests were collected at three days intervals and the viral titres of each viral harvest were quantified by Florescent Antibody Test (FAT). In general the second viral harvest had higher titre than others which was aliquoted and used as working seed.

2.2.4. Sterility Assay

Sterility of Vero cell working cell bank, rabies virus working seed lot were ensured by inoculating into 3% SCDM and observed for fortnight at 37 °C.

2.2.5. Mycoplasma Screening

The PCR technique was employed for effective monitoring of mycoplasma contamination in the starting materials like Virus, cell substrate, tissue culture medium and fetal bovine serum (FBS). The template DNA isolated from samples by thermal shock lysis followed by phenol : chloroform : isopropyl alcohol (25:24:1) extraction, ethanol precipitation and dissolved in nuclease free water. Two set of primer : MCGp F1, MCGp R1 primer pair target rRNA of 16S and MCGp F2, MCGp R2 primer pair target the 23S genes respectively subjected for amplification in 20 µl reaction. The amplified product were resolved in 1% agarose gel and visualized under the UV Gel documentation system in comparison with positive and negative control reactions.

2.2.6. Culturing Technology

The bioreactor culture for rabies virus (pv-11) propagation was standardized in parallel with roller bottle culture using Vero cell host system. In this study the Vero cell propagation using two different media in both the culturing methods and the resulting viral titre were compared with each other.

2.2.7. Vero cell revival and subculture

The frozen Vero cells (passage 143) thawed quickly and the cells were washed once with pre-warmed MEM Eagle's revival medium to remove the DMSO. The Vero cells were incubated with MEM Eagle's revival medium at 37 °C in 5% CO_2 incubator for 5 days during which at the end of 2nd day incubation the culture is replenished with fresh MEM Eagle's growth medium. Confluent monolayer's of the Vero cells were washed twice with sterile PBS and trypsinized with pre-warmed 0.25% Trypsin. The cells were resuspended with fresh growth medium and subcultured into new tissue culture flasks based on the cell count.

2.2.8. Cell count

$$C = \mathbf{n} \times \mathbf{v} \times 10^{-4}.$$

Where,
C = cell concentration in cell suspension/ml
n = average number of cells/mm^2 area.
v = dilution factor
Total cell per ml (C) = average cell count X dilution factor X 10^{-4}.

$$\text{Viability \%} = \frac{\text{No. of live cells counted}}{\text{Total No.of Cells (Live \& Dead)}} \times 100$$

2.2.9. Vero cell propagation for Bioreactor and Roller bottle culture

The cells were subcultured from passage 143 to 148 as illustrated in figure 1

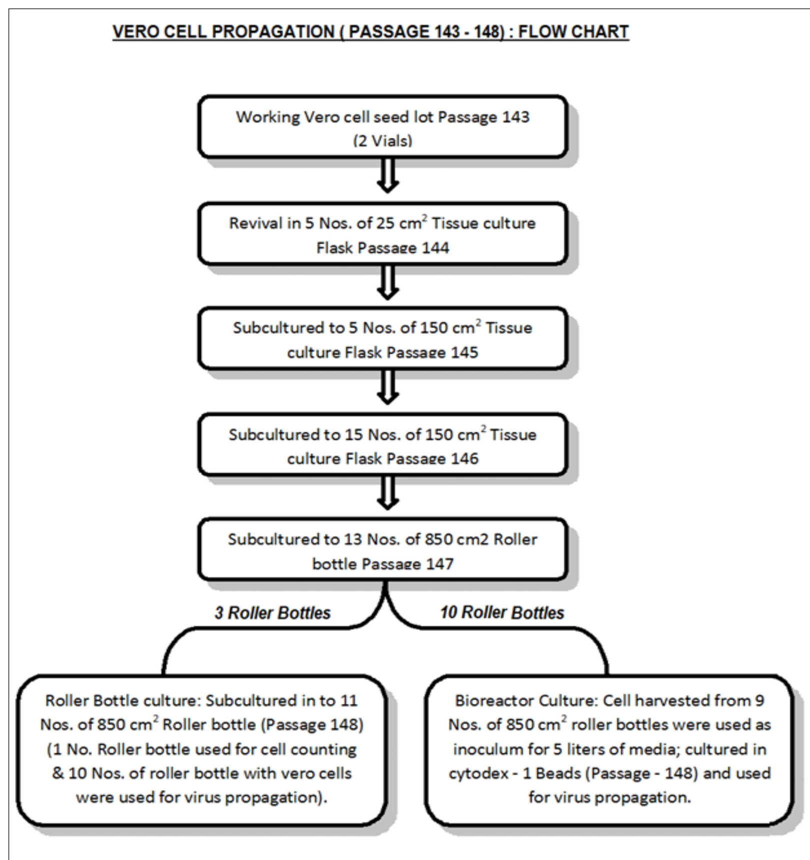

Figure 1 Flow of Vero cell propagation in different passage levels

2.2.10. Vero cell adaptation to RPMI 1640 medium

The Vero cells were subcultured in MEM Eagles growth medium (passage 145) and subjected for growth up to 48 hours. After that fresh RPMI 1640 was replenished and cell grown at 37 °C incubator with 5% CO_2 until the formation of complete monolayer. The adapted cell subculturing was performed from passage 145 to 148 as per the flow chart with RPMI 1640 medium and used for rabies virus propagation.

2.2.11. Roller Bottle Culture

Vero cells were trypsinized from 150 cm^2 tissue culture flasks, 42 x 10^6 cells were seeded in to each of 850 cm^2 roller bottle along with 200 ml of the growth medium and placed in roller apparatus at 37 °C hot room. The speed of the roller was kept at 25 rotation per hour for initial 24 hours and increased to 40 rotation per hour until the completion of 5 day incubation. During the third day incubation the growth medium was replenished with fresh growth medium. The cells were subjected for rabies virus propagation in roller bottle /or subcultured into fresh roller bottles for further passages /or used as inoculum for Bioreactor culture.

2.2.12. Viral infection in roller bottle

The required quantity of seed virus for the infection of roller bottles was calculated using the following formula:

$$\text{MOI} = \frac{\{(\text{Antilog of the virus titre used for infection: A}) \text{ X } (\text{Volume of the virus pool used for infection: V})\}}{(\text{Total number of cells: n})}$$

$$V = \frac{(\text{MOI X n})}{A}$$

The MOI (multiplicity of infection) 0.2 and 0.3 were used for infection of roller bottle pertaining to virus seed preparation and batch culturing respectively. The volume was made upto 20 ml using sterile growth medium and added to each roller bottle. The bottles were tightly caped, placed in roller apparatus for virus adsorption for about 90 minutes in 37 °C hot room and continued the incubation with 200 ml of fresh growth medium.

2.2.13. Viral harvest from Roller bottle

Four viral harvest were collected at three days intervals (3rd, 6th, 9th and 12 days) and immediate after each viral harvest, 200 ml of fresh maintenance medium was replenished.

2.3. Bioreactor culture

2.3.1. Preparation of Bioreactor

The Bioreactor and addition lines were washed with WFI and calibrated pH, DO probes were fixed in appropriate place. Four litres of PBS (pH 7.4) was added to the vessel and it was ensured that the pH, temperature and DO probes were immersed. The exhaust line of the bioreactor was connected with two numbers of 0.22µ air filters and kept open during sterilization. The tubing's of medium addition lines, cell inoculum line, virus infection line, harvest lines were properly arranged to maintain the sterility throughout the culturing process. The bioreactor was placed in autoclave and sterilized at 121 °C for 45 minutes and bring down to 37ºC by connecting to control panel. Prior to the Vero cell culture, the sterility maintenance and operation of the bioreactor was ensured using simulated run with 3% soybean casein digest medium (SCDM) up to fortnight.

2.3.2. Cytodex - 1 Microcarrier beads processing

The micro-carrier cytodex - 1 (GE Healthcare) was swollen overnight with sterile Ca^+ and Mg^+ free PBS in a siliconized container. The beads were washed twice with sterile fresh PBS and sterilized by autoclaving at 121 °C for 15 minutes. The supernatant was removed aseptically and three washes were made with respective growth medium (MEM / RPMI 1640) to remove the PBS completely and stored at 2 – 8 °C until its use.

2.3.3. Bioreactor culturing

Five litre batch cultures were performed for both MEM Eagles and RPMI 1640 media cultures. The Bioreactor was prepared for batch culturing as described above, the PBS in the vessel was drained aseptically and 3 litres of sterile completed growth medium along with required quantity (3 grams / litre) of cytodex -1 micro-carrier beads were added

to the vessel. The bioreactor was connected with air inlet line, alkali solution with peristaltic pump, temperature probe, exhaust line, pH probe, DO sensor and jacket water lines to the control panel. The signals from each probe were ensured in the HMI (Human machine interface) before starting a batch. The cell culture parameters such as 37 °C temperature, agitation by impeller with 18µm spin filter at 60 rpm, 0.2 litre / minutes of gas sparging, pH at 7.2, DO level at 55% saturation [1, 6, 7, 8] were fixed and allowed for at least 8 - 12 hours to get stabilized before the Vero cells inoculation. Pure air, pure Oxygen and pure carbon dioxide were sparged into the culture through the 0.22µ filter mediated by automated loop mediated control system. 8.8% sodium bicarbonate buffer was in place to adjust the pH reduction during entire process and operated through auto mode.

The Vero cell in the roller bottles were trypsinized and the cells were enumerated using Haemocytometer. 3.5×10^8 cells were inoculated to each litre of culturing volume and the volume was made up to 4 litre with the respective sterile completed growth medium. During the adsorption period of six and half hours the agitation of the bioreactor was switched off for 20 minutes to facilitate the adsorption of Vero cells on the cytodex - 1 beads and intermittently switched on for 5 minutes to mix the cell with cytodex beads evenly [9, 10].

2.3.4. Vero cell propagation

The culturing conditions were maintained as per the set parameters mentioned above. On third day of Vero cell growth phase, 3 litres of growth medium was replenished in the bioreactor to facilitate the availability of nutrients for cell growth. The sample was collected from the bioreactor on daily basis to observe the cell growth. The sample was transferred aseptically to the $25cm^2$ tissue culture flask and visualized for cell adsorption / growth under the BIO-RAD cell imager.

2.3.5. Virus infection

Sample was collected on fifth day from the bioreactor and complete monolayer of the Vero cells on the cytodex -1 micro-carrier beads was ensured. The cells per ml of the culture broth were calculated by trypsinizing cells from the cytodex beads followed by cell counting procedure. Based on the cell count the required quantity of working virus stock was calculated and infected to the grown Vero cell in the bioreactor at the concentration of 0.3 MOI [1, 6]. The set parameters such as Temperature, pH, DO were adjusted to 34 °C, 7.4 and 40% (±5%) saturation level respectively after the infection for virus propagation [1] and maintained the same until 4th harvest.

2.3.6. Viral harvest from bioreactor

The agitator was switched OFF and the beads were allowed to settle for five minutes. The 4 litres of the viral fluid (supernatant) was collected aseptically in a sterile container and fresh maintenance medium was replenished with the equal volume of the harvest after every viral harvest. The entire volume of five litres along with the micro-carrier beads were collected on fourth viral harvest by the dedicated collected port and the beads were separated from the viral fluid.

2.3.7. Viral Titration of roller bottle and bioreactor viral harvests

The viral titres in each medium (MEM eagles / RPMI 1640) in response to the culturing methods (roller bottle / bioreactor) were quantified by in-house standardized real time PCR technique. The viral mRNA from the samples were isolated and converted in to cDNA by Biorad iscript cDNA synthesis kit. The cDNA were subjected to real time PCR amplification with ATGGAACTGACAAGAGACCC (forward), GTGCTTTGCCCGGATATTTT (reveres) primers pairs using SYBR green master mix in 20µ reaction volume. The viral titre in each harvests were quantified in comparison with quantitative standard included in every assay [11].

3. Results and discussion

3.1. MEM and RPMI 1640 medium

The MEM Eagles medium was routinely used for the rabies vaccine manufacturing. In this study the RPMI 1640 medium was used along with MEM Eagles medium to compare each other for Vero cell and virus propagation in roller bottle and bioreactor culturing methods. The medium were prepared in tissue culture grade water and sodium bicarbonate was used for the buffering activity to stabilize the pH variation of the medium due to cellular excretion. The sterility of the media batches were ensured using 3% SCDM before subjecting for media completion (Revival/Growth/Maintenance medium) since the antibiotic to be added for medium completion which makes the false negative in case of any contamination in the tissue culture medium.

3.2. Working Vero cell bank and virus seed lot system

To establish the working Vero cell bank, the cell at passage 140 revived in 25cm^2 tissue culture flasks and grown for the period of five days with an intermittent media change. The cells were trypsinized and enumerated using Haemocytometer. The cell propagation and viability percentage in each passage of Vero cell working cell bank preparations were enumerated. An average of 10.7 X 10^6, 45.32 X 10^6, 220.4 X 10^6 were propagated in 25cm^2 TC flask, 150cm^2 TC flask and 850 cm^2 Roller bottle respectively with about 96~98% cell viability during working cell bank and virus seed lot preparation. Working cell bank was prepared by pelletizing the harvested Vero cells with 600g centrifugal force and resuspended in the concentration of 10 million cells per ml of freezing media mixture contain 40% fetal bovine serum, 50% MEM eagles, 10 % of DMSO and distributed in to cryo vials aseptically. The Vero cells in the passage 143 were frozen using stratacooler and stored in the liquid nitrogen vapour phase as working cell bank for further propagation for this whole study. The working virus seed lot prepared in passage 145 by infecting with 0.2 MOI of virus in roller bottle culture. The yield of rabies viral particle: harvest-1 Log 5.375, harvest-2 Log 6.875, harvest-3 Log 6.250 and harvest-4 Log 5.875. The viral harvest 2 containing higher viral titre (Log 6.875 = 7498942 particle /ml) was aliquoted in to multiple vials each contain 45 ml and stored in -80 °C freezer as working virus seed lot.

3.3. Seed Sterility observation

The sterility of the Vero cell working cell bank, virus seed lot were tested to ascertain its sterility before proceeding with culturing activity using these seed materials. There were no turbidity observed upon completion of fortnight incubation period in the sample inoculated with 3% SCDM and it ensures that our seed lot preparations are free from bacterial and fungal contamination. Both positive and negative control medium was included in each set of testing procedure to validate the assay.

3.4. Mycoplasma detection

The mycoplasma is the common contaminants of the tissue culture laboratory and it is essential to test the starting materials to prevent the contamination during the tissue culture procedure. The mycoplasmas are small prokaryotic organisms without any rigid cell wall because of this property it can pass through the membrane while doing the sterile filtration [12]. The materials such as cell seed, virus seed, tissue culture medium, fetal bovine serum [13] were screened qualitatively for the mycoplasma contamination by polymerase chain reaction before initiating any activity. The detection of mycoplasma through PCR is sensitive, rapid and suitable method for the cell culture facility [14].

This primer pairs can able to detect the species of mycoplasma, (*Mycoplasma fermentans, Mycoplasma hyorhinis, Mycoplasma arginini, Mycoplasma orale, Mycoplasma salivarium, Mycoplasma hominis, Mycoplasma pulmonis, Mycoplasma arthritidis, Mycoplasma neurolyticum, Mycoplasma hyopneumoniae,Mycoplasma capricolum*) and one species Ureaplasma (*Ureaplasma urealyticum*) with specific amplified product for each species. Two positive controls (PC-1 : 810 bp and PC-2 : 590 bp) and a negative control were included in every assay setup and it passed the validity criteria. There was no specific amplification of the product observed on samples, it indicates that there was no mycoplasma contamination found in our Vero cells, viral seed, fetal bovine serum, tissue culture medium were used.

3.5. Vero cell revival

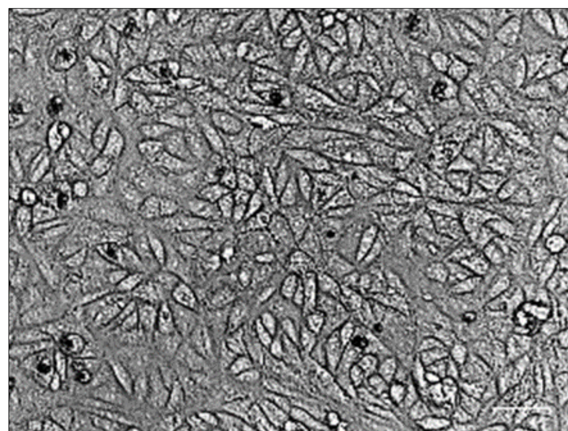

Figure 2 Vero cells with dead cells at revival stage (24 Hours)

Two frozen vials of Vero cells at the passage143 containing 10 million Vero cells per ml were revived using the revival medium. During the 24 hours of incubation at revival stage, it was observed that few dead cells (Figure : 2) were suspending in the cell culture fluid which were removed by replenishing the fresh pre-warmed Eagles medium (growth medium)and incubated further for growth. A study revealed that the viability of the Vero cells with various percentage of the DMSO in the cryopreservation medium and they stated maximum of 65% viability attained in the vials stored with 10% of DMSO in the cryopreservation medium [15]. It was noticed that the pH reduced to acidic condition during 3rdday of Vero cell propagation, hence the fresh growth medium replenishing procedure was followed at the end of the 3rd day of incubation. On the 5th day of incubation the confluent monolayer was formed in the tissue culture flask. The Vero cells were propagated (from passage 143 - 148) to culture eight batches which include four roller and four bioreactor batches with two different medium. Total number of Vero cells, viability percentage in each passage were calculated by cell counting procedure and the results were tabulated in Tables 1.

Table 1 Cell counting of MEM Eagle's Medium

	MEM Eagle's Batch 1		**MEM Eagle's Batch 2**		**RPMI 1640 Batch 1**		**RPMI 1640 Batch 2**	
Sample ID / Details	**Cells in million**	**Viability %**	**Cells in million**	**Viability %**	**Cells in million**	**Viability %**	**Cells in million**	**Viability %**
Passage - 144 (Seed Revival) (5 Nos. 25cm^2 TC Flask)	56.4	96.34%	54.6	95.89%	57.57	97.11%	56.28	96.74%
Passage 145 (5 Nos. 150 cm^2 TC Flask)	204.39	97.35%	195.5	97.76%	153.0	96.73%	163.32	97.48%
Passage 146 (15 Nos. 150 cm^2 TC Flask)	645.12	97.76%	674.1	96.88%	593.63	97.37%	624.15	98.09%
Passage 147 (3 Nos. 850 cm2 roller culture)	710.01	98.22%	691.02	98.32%	674.12	98.02%	696.92	98.36%
Passage 148 (1 roller for Vero cell count & MOI reckoning)	228.8	98.58%	224.07	98.82%	221.1	98.75%	224.2	97.75%
Passage 147 Bioreactor (10 roller to 5 Litres bioreactor)	2228.2	98.54%	2352.0	98.83%	2278.0	98.89%	2306.6	98.76%
Passage 148 Cytodex -1 (1 ml for virus infection)	8450	97.04%	8750	97.71%	8500	96.47%	8350	97.00%

3.6. Vero cell sub culturing and inoculum

4 million cells per 25cm^2 tissue culture flask are seeded at the point of revival stage since the viability of the cryo preserved cells are much low compared to normal passage. Further passages of the Vero cells were performed with constant quantity of inoculums and medium. The average cell count and propagation ratio in respect of specific containers / culturing methods are specified in table 2.

Accumulation of lactate and Ammonia and higher quantity of glucose consumption in the medium were perceived within initial 4 days from post inoculation of Vero cells [5, 6, 7, 8]. Since the fresh medium replenished on every third day of incubation to keepup the nutrients, growth factors to the cells and maintain the pH in the optimal level for both roller bottle and bioreactor culturing methods.

Table 2 Cell propagation ratio in different culturing methods

Culturing area & Culturing Methods	Vero cell seeding quantity	Volume of medium	Avg. Cell count	Avg. Vero cell Propagation ratio
25cm2 TC flaks (revival)	4 x10^6	15 ml	11.24 x10^6	1 : 2.8
150 cm2 TC flaks (subculture)	8 X 10^6	100 ml	41.98 x10^6	1 : 5.2
850 cm2 roller bottle	42 x 10^6 / roller bottle	200 ml	1.12 x 10^6 / ml	1 : 5.3
Bioreactor culture	3.5 X 10^8 / litre	5 litres	1.70 x10^6 / ml	1 : 4.8

3.7. Vero cell adaptation in RPMI 1640 medium

During the Vero cell adaptation with the RPMI 1640 medium it was observed that the cell proliferation rates get decreased. In passage 145 it was enumerated that an average of 199.94 x10^6 cells propagated in MEM Eagles medium and in RPMI 1640 an average of 158.16x10^6cells were propagated respectively in two different batches of each medium. It was calculated approximately 20 % lesser growth rate in the RPMI 1640 medium during its adaptation. Due to low propagation rate, the split ratio of the MEM Eagles passages were also reduced deliberately as equal to RPMI 1640 media passages appropriately. It was observed that, the cell propagation ratio got increased gradually in the next consecutive passages 146 and 147 and found equal growth rate in RPMI 1640 medium in passage 148.

3.8. Roller bottle cell growth

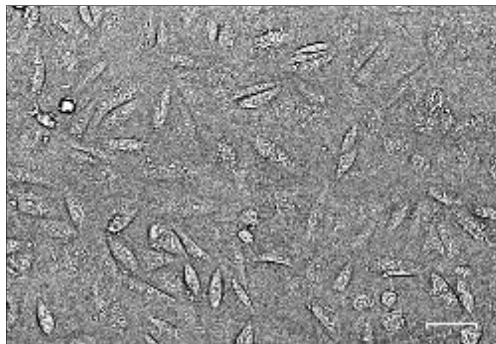

Figure 3a Vero cell growth on 24 Hours

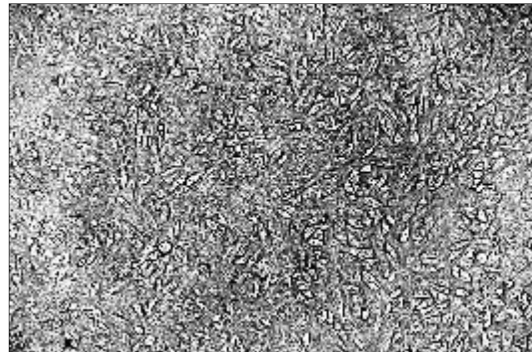

Figure 3b Vero cell growth on 3rd day

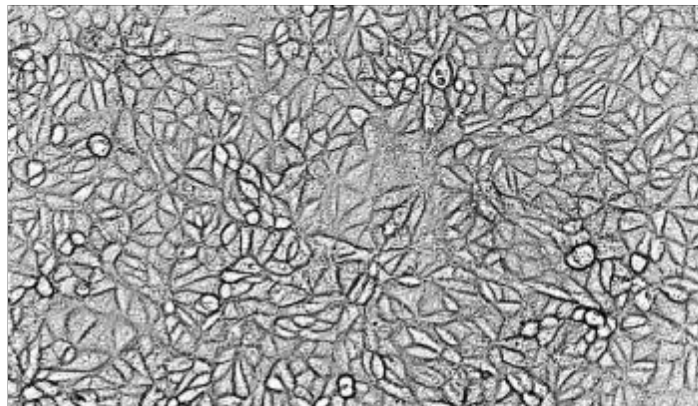

Figure 3c Vero cell monolayer on 5th days of Incubation

Figure 3 Vero cell observation in Roller bottle culture

The pH of the culture medium in the tissue culture roller bottle were measured at the end of 3rd day during the Vero cell growth. The measured pH range between 6.0 to 6.5 in the MEM Eagles medium and in RPMI 1640 medium between the

ranges of 5.7 to 6.3. Higher rate of pH reduction was observed in RPMI 1640 medium than the MEM Eagles medium. The roller bottles were visualized under cell imager to ensure the Vero cell growth at 24 hours, 3rd day and 5th day of post inoculation (figure 3.a, 3.b & 3.c). The Vero cell obtained in a roller bottle using MEM Eagles medium were ranging between 222.82x 10^6 cells to 236.67x 10^6 cells and in RPMI 1640 between 227.80x 10^6 cells to 230.66x 10^6 cells in passage 147.

3.9. Bioreactor Culturing Method

3.9.1. Bioreactor Culturing

The trypsinized Vero cells were transferred to Bioreactor vessel along with cytodex - 1 micro-carrier beads. The sample was collected after the six and half hours adsorption period and it was observed under the cell imager for even mixing and attachment of the Vero cells (Figure 4.a). The culturing process was continued with auto mode and the optimal growth parameters were maintained as specified in the procedures. The pH of the medium was maintained with 8.8% sterile sodium carbonate buffer placed in appropriate addition line and it takes approximately 25~30 ml of carbonate buffer per day. The pH & DO were the critical set parameters for the growth of Vero cells and maintained at 7.2 & 50±5% saturation point respectively. To maintain the pH and DO in the set parameter, the sparger gas flow was intermittently switched ON for 2 minutes and OFF for 1 minutes through loop mediated control system.

The ratio between the Vero cells and cytodex - 1 beads concentration is one of the crucial factor to get optimum growth of Vero cells on the micro-carriers. 200 million cells per litre of the culture medium was inoculated during optimization stage and it was observed only partial adsorption on the micro carrier beads. Further the cell concentration was increased to 350 million cells per litre to get the optimal growth. The growths of the cells were monitored on daily basis (Figure 4.a, 4.b, 4.c, 4.d). The Vero cells obtained from the bioreactor culturing method in the range of 1.69 to 1.75 million cells / ml of culture by MEM Eagles medium and 1.67 to 1.70 million cells / ml by RPMI 1640 medium. The average quantity of Vero cells in 10 roller bottles (2 litres) were in the concentration of 1.12 x 10^6 cells per ml of culture and in the bioreactor (5 litres) it is in the range from 1.70 x10^6 cell per ml of culture at passage 148. It is estimated that, about 35% increase in cell quantity in bioreactor system than the roller bottle culturing method.

3.9.2. Virus Infection

0.3 MOI of virus infection was kept as common for Bioreactor and Roller bottle culture with two different medium to find out the differences between the culturing methods and medium on viral titre. The viral seed lot that with the viral titre log value of 6.875 (7498942 virus particles / ml) was used as inoculum for all the culture. The required quantity of the viral seed stock was calculated based on cell count enumeration in the respective culturing methods. One roller bottle was used for enumeration of cell count in roller bottle culturing method. For bioreactor culture 1 ml of the cytodex - 1 beads along with Vero cells were trypsinized and total cell count was calculated for virus infection (Figure 4.e). In post infection period the exhaust air from the bioreactor was sparged through 5% sodium hypochloride solution for chemical inactivation since there is the chance to release the virus particle through this.

3.9.3. Viral Harvest

Four viral harvests were collected from each batch. The fourth viral harvest had more Vero cellular debris than the other three viral harvests and it was removed by centrifugation (Figure 4.f). The Vero cellular debris are the main interfering components during downstream concentration and purification processes and it needs to be removed for easy operation. The sterility test results ensured that the harvests were sterile and stored at 2 - 8 °C until further downstream process.

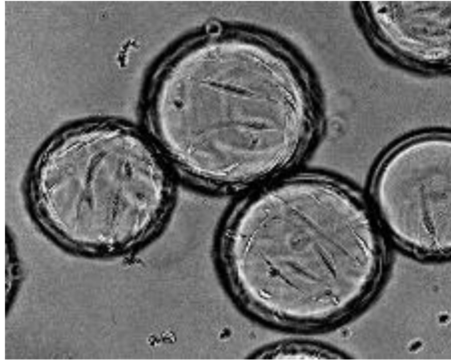

Figure 4a initial attachment

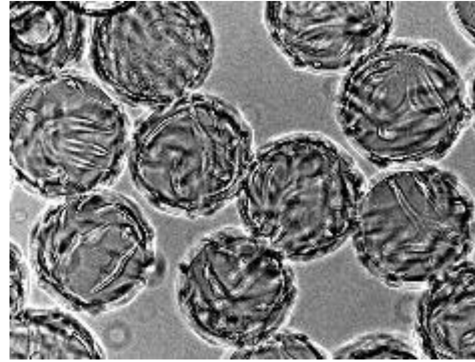

Figure 4b 1st day of growth

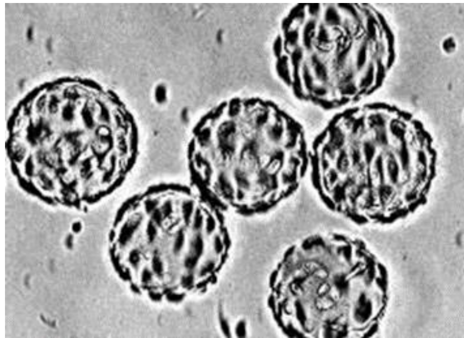

Figure 4c Third day of growth

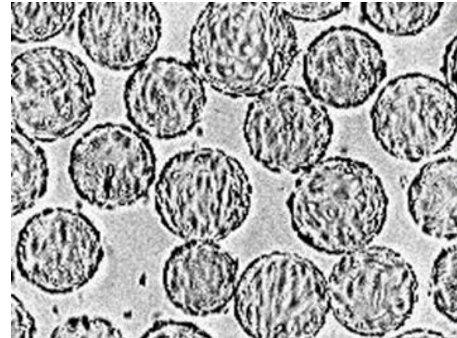

Figure 4d 5th Days of growth

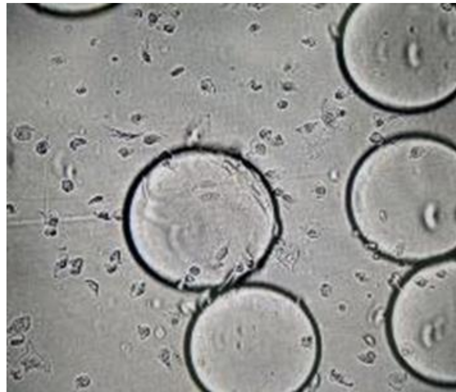

Figure 4e Trypsinized Vero cells from Cytodex -1 beads

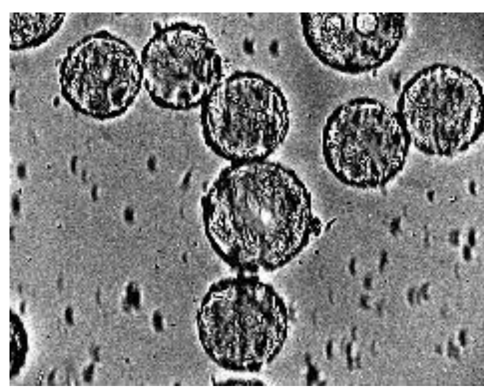

Figure 4f Vero cell on Cytodex during 4th Harvest

Figure 4 Vero cell observation on cytodex - 1 microcarrier beads in different stages bioreactor culture

3.9.4. Rabies Virus titration

The in-house standardized Real Time – PCR technique was used as alternative tool for quantification of rabies virus PV -11 strain and it express the viral titre in copy number in each viral harvests. The method was standardized in comparisons with Mouse inoculation test (MIT) and Fluorescent Antibody Test (FAT) and described in our other study [11]. The RT-PCR quantification method is rapid other two methods and use of laboratory animal can be avoided by applying this. The viral titre quantified by real time PCR ranging from Log 6.069 to Log 7.106 in MEM Eagles and Log 6.044 to Log 6.827 in RPMI 1640 by roller bottle culturing method. The titre in the bioreactor culture ranging from Log 6.559 to Log 7.216 in MEM Eagles and Log 6.498 to Log 7.169 in RPMI 1640 medium. Expressing the viral titre in copy number will show the exact proportion than the log value. Example, log 6.5 (copy number 3162277) is three fold higher viral titre than log 6.0 (copy number 1000000). With reference to the technical report series of rabies vaccine preparation by WHO, the rabies viral harvest should contain the viral titre greater than or equal to the log 6.0 [16, 17] and all our viral harvest met their recommendation. The viral titre in each of the harvests with respect to culturing method & respective medium were shown in table 3. The average viral copy number of two identical batches obtained by real time PCR were plotted in figure 5.

Table 3 Rabies viral titration by real time PCR in viral harvests of roller bottle culture and bioreactor culture

Medium	**MEM Eagles**		**RPMI(1640)**	
Culturing method	Batch 1 Viral Titre in log	Batch 2 Viral Titre in log	Batch 1 Viral Titre in log	Batch 2 Viral Titre in log
Roller bottle culture	6.369	6.601	6.194	6.345
	6.691	7.106	6.513	6.852
	6.656	6.598	6.366	6.695
	6.069	6.421	6.044	6.456
Bioreactor culture	6.729	6.695	6.679	6.863
	7.099	7.216	7.169	7.043
	6.809	6.886	6.899	6.957
	6.559	6.661	6.619	6.498

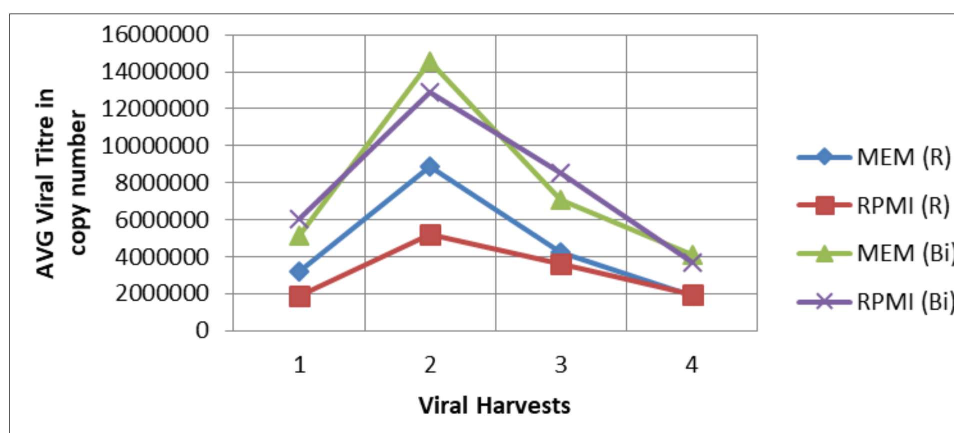

Figure 5 The Average rabies viral titre in copy number corresponding of log value

4. Conclusion

Screening of working cells bank and working viral seed, serum for bacterial, fungal and mycoplasma are inevitable in animal cell culture. Both MEM Eagles and RPMI 1640 mediums perform equally but the culturing method plays a vital role in the virus propagation in the Vero cells. The bioreactor system yields more viral titre than the roller bottle culture irrespective of MEM Eagles or RPMI 1640. In the biological system, there is negligible difference in the cell count, viral titre in the identical batch itself. In a batch the second harvest always gave higher yield than the other viral harvests and the same was reflected in bioreactor culturing method also. In the mass production, the viral titer in each harvests is directly proportional to the final vaccine yield.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest in this Research article.

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