Original Research Article

Comparative analysis of MEM, RPMI 1640 medium on rabies virus propagation in Vero cells and virus quantification by FAT

ABSTRACT

Rabies disease can be preventable through vaccination is the only way for effective control and the vaccine is administered as post exposure prophylaxis method (PEP) along with rabies immunoglobulin. For the preparation of the vaccine, the high titre rabies virus has to be propagated in suitable host system like vero cells. In this study, the virus was propagated in tissue culture flask in two different tissue culture media namely MEM and RPMI-1640, keeping Vero cell line as the host system. This preliminary study is to find the media that yields a better viral titre as high, the influence of harvesting intervals on viral titre for the reason that the vaccine yield is directly proportional to the viral titre during the virus propagation stage and leads to cost effective vaccine. The Vero cell was revived from passage 154 and the part of cells were subjected for adaptation in RPMI-1640 media with gradual media replacement. The passaging of RPMI 1640 media cells were continued until it reaches the equal cell count of MEM (p-158). The confluent monolayer of Vero cells was maintained in the same passage level with appropriate media. The cell count of MEM media, RPMI-1640 were 10.28×10^6 and 10.35×10^6 respectively in $25cm^2$ tissue culture flasks. The Vero cells were sub-cultured in 175 cm^2 tissue culture flasks infected with 0.2 MOI of virus and the viral titration was estimated through FAT test (Fluorescent Antibody Test). The highest viral titre obtained from RPMI-1640 media batch ($10^{-6.125}$/ml) and MEM media batch ($10^{-6.25}$ /ml). The two days interval viral harvests shows (Batch 1 & 3) the viral titre log ranged between $10^{-4.375}$ to $10^{-5.875}$ and three days interval harvests (Batch 2 & 4) the viral titre log ranged between $10^{-3.750}$ to $10^{-6.250 \text{ per mL}}$.

KEYWORDS*: Cell culture, FAT test, MEM, Rabies virus, RPMI-1640, Vero cell.*

1. INTRODUCTION :

Rabies disease is a highly fatal viral zoonosis caused by rabies virus belonging to the Lyssavirus family affecting the central nervous system which is transmitted to humans from the infected saliva of a rabid animal. The characteristic symptom of the disease is 'hydrophobia' commonly known as fear of water [1, 2]. Although human rabies is eradicated from most of the developed countries, it still prevails to cause many deaths in many developing countries [3]. There is no cure for the rabies disease after the symptom development, the result is always fatal. Since there is no known effective treatment, preventive measures are the only way to combat the disease and this is achieved by effective vaccination [4].

The vero cell line is the most widely accepted continuous cell line by regulatory authorities and has been used for over 30 years for the production of polio and rabies viral vaccines [5]. The vero cells are considered non-tumorigenic below a certain passage number and safe to use as a substrate for vaccine production [6].

To obtain the inactivated form of the vaccine the virus has to be grown, harvested, concentrated, inactivated, purified and formulated with preservative. Unlike the earlier methods like animal systems and embryonated eggs [7], cell culture technology is widely used for the propagation of virus due to its various advantages like avoiding the use of animal models by implementing 3R's principles. Non-tumorigenicity and infinite division of continuous cell lines for virus culture and propagation under laboratory conditions makes it feasible. The vero cell line is a continuous, adherent cell line established from African green monkey's kidney cells is the most widely accepted cell line by WHO since it is highly productive and many viruses can be grown in vero cells [6, 8, 9].

2. MATERIALS AND METHODS:

2.1 **Cell line:** Vero cell line at passage 154 (obtained from ATCC CCL-81), was used as host system for rabies propagation and BHK-21 cell line at passage 143 (obtained from IVRI, Bangalore) was used for the determination of viral titre through FAT test.

2.2 **Virus strain:** Vaccine strain of Rabies virus PV 11(Pasteur Virus 11) that is routinely used for the vaccine manufacturing process was used in this study citation or proof.

2.3 **Culture media:** Minimum Essential Medium (MEM: HiMedia; Cat # AT047), with 2.2g $NaHCO_3$/L and Roswell Park Memorial Institute Media (RPMI-1640: HiMedia; Cat # AT028), with 2.0g $NaHCO_3$/L were used in this study. Both the media are supplemented with 0.5% Amphotericin and 1% Neomycin. The media was filter sterilised with 0.22 µ rapid flow disposable filtration unit. The concentration of Fetal Bovine Serum (FBS-Gibco) varies for revival, growth and maintenance media with 10%, 5% and 2% respectively. For the growth of BHK-21 cell line, Eagle's MEM media supplemented with 10% TPB, 10% FBS, 1% Neomycin and 0.5% Amphotericin wqas used.

2.4 **Vero Cell Revival, Culture Technology and Maintenance:** Cryopreserved vero cells in the passage 154 was revived in 25 cm^2 vented tissue culture flask using the revival media after the removal of cryopreserving agent (DMSO) from the cell seed [10,11] and incubated at 37ºC with 5% CO_2 for 5 days. The cells are observed on daily basis to check the confluency and is there any contamination. The cell confluent monolayer was splitted and seeded into fresh tissue culture flask in 1:4 split ratio for further passaging.

2.5 **Adaptation and Maintenance of Vero cells in RPMI-1650 media:** To adapt the vero cells in a different medium other than its original base medium, the cells are exposed to the new medium gradually and media replenishing was given on daily basis. The percentage of each medium on different days of adaptation period at passage level 155 was done as follows:

Table 1: **Adaptation of Vero cells in RPMI-1640 media**

Days	% of RPMI 1640 Media	% of MEM Media
Day 0	Nil	100%
Day 1	25%	75%
Day 2	50%	50%
Day 3	75%	25%
Day 4	100%	Nil

The cells maintained in both media are passaged further in respective media until the RPMI 1640 cells had reached more than 95% vero cell quantity in comparison with MEM medium. Further the vero cells were sub-cultured into 175 cm^2 tissue culture flask for viral infection.

9 nos of 175 cm^2 vented tissue culture flasks of each media (RPMI 1640 and MEM) containing confluent monolayered vero cells in the passage level 159 were subjected for viral infection and a flask from each media was used for cell count using haemocytometer to calculate the viral inoculum required for infection with 0.2 MOI.

2.6 **Viral Infection and Harvest:** The calculated volume of virus seed for each flask was diluted with respective media up to 20 ml added to flasks aseptically and kept at 37ºC and 5% CO_2 incubation for 90 minutes. The virus solution spreading over the surface was done at every 30 minutes intervals to enhance the virus attachment on vero cells. After the virus adsorption period, the virus solution in each flask were discarded and 60 ml of freshly prepared respective media (maintenance media) added to each flask. 8 flasks in each set of media were further divided into two batches. Each contains 3 flasks and a control flask. As a result, we had 4 different batches of tissue culture flasks with two different media. The MEM medium tissue culture flasks were marked as Batch 1, Batch 2 and RPMI 1640 tissue culture flasks were marked as Batch 3, Batch 4. The Batches 1 & 3 were harvested at every 2 days interval and batches 2 & 4 were harvested at 3 days interval up to 5^{th} viral harvest.

2.7 **Viral titre estimation using FAT test :** Viral titre estimation was done using Fluorescent Antibody Test, FAT [12] using BHK-21 cell line as host system. 20 viral harvests were obtained from 4 different batches and each sample was serially diluted with BHK 21 growth medium in the dilution range of 10^{-1} to 10^{-6}.

BHK-21 cells in the Passage 158 that were grown in 25cm^2 tissue culture flasks were washed with sterile pre warmed PBS, detached with trypsin-EDTA and 13 ml of homogenized suspension with BHK-21 growth medium as host system. 0.1 mL of Each diluted sample was mixed with 0.1ml of BHK-21 growth medium in 96 well plate as per test layout. 50µL of the BHK-21 cell suspension containing 20,000~25,000cells and was added in each well. Each plate included with cell control and a virus control that serves as standard during the enumeration of the viral particle. The plates

were covered and incubated at 37°C with 5% CO_2 for 48 hours. The fluids were removed and the cells were fixed by the addition of 70% acetone in PBS and the plate was incubated at -20℃ for 30 min. After fixation, freshly prepared 50µl of Anti-Rabies FITC antibody conjugate was added to each well and incubated at 37℃ for 30-40 mins. The fluid was then removed and the plates were washed twice gently with Distilled water. A drop of glycerol carbonate bicarbonate buffer of pH 9.0 was added to each well as mounting buffer. The plate was then read using a fluorescent microscope and the virus quantity in each sample was obtained by calculation using Spearman-Karber method.

3. RESULTS AND DISCUSSION

3.1 Vero Cell revival and Maintenance : Cryopreserved Vero cell in passage level 154 was revived using the MEM revival media. Frozen cell stock containing cryo-preservant dimethyl sulfoxide (DMSO) which is harmful to the cells and it is necessary to dilute and remove before transferring the cells for revival [11]. The media in the tissue culture flasks was replenished during 24 hours of incubation to remove the dead cell. Further the cells were splitted into 1:4 ratio (approximately 2.5 million cells) to get optimum vero cell monolayer. Complete monolayer of the vero cells were observed on the 5th day of incubation (Figure 1.b). The number of cells in each TC flasks was enumerated using hemocytometer cell count procedure at each passage level. The cells were passaged in MEM for the MEM (batch 1&2) and portion of vero cells were adapted in RPMI 1640 media for RPMI (batch 3 & 4).

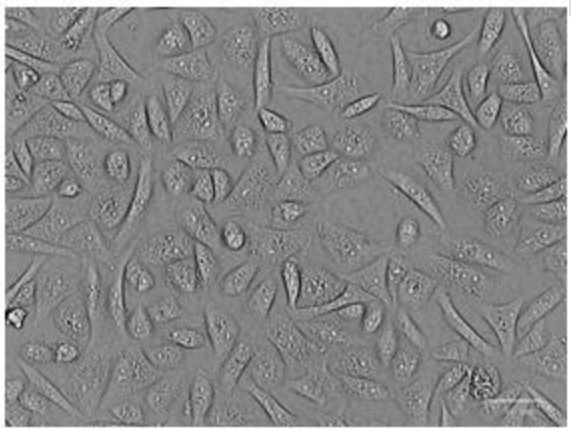
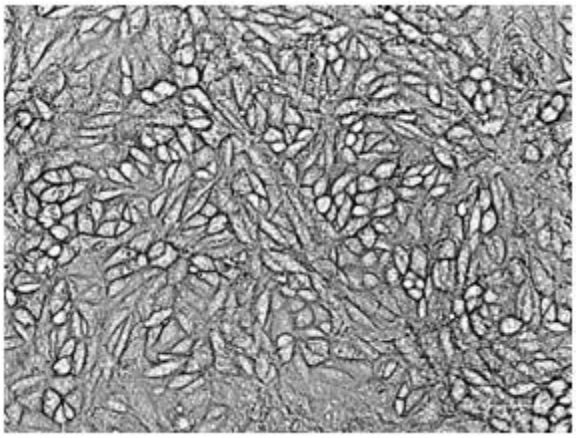


Figure 1.a : Vero cells at 24 hours



1.b : Vero cells at 5th Day

3.2 **Adaptation and Maintenance of Vero cells in RPMI-1650 media :** While adapting the Vero cell to RPMI 1640 media, slow growth of Vero cells was observed due to the exposure to new growth environment and the cells takes time to adjust to those conditions. The change in growth rate was observed and calculated based on cell propagation ratio on each media. The P-155 batch of MEM media gave a yield of 10.05 x 10^6 cells whereas newly adapted P-155 batch of RPMI-1640 media

gave a yield of 7.56 x 10^6 cells and there is ~25% of reduction in RPMI 1640 during initial stage of adaptation. The cell line is passaged further 3 times from passage 156 to 158 for complete adaptation and the cell enumeration results shows that the RPMI 1640 cell count as equal to the MEM media [13]. The cell count in RPMI 1640 media was 10.35 x 10^6 and in MEM it was 10.28 x 10^6 cells per 25 cm^2 tissue culture flasks at passage level 158 in respective media. The figure 2 illustrates the cell count on each passage level with MEM & RPMI 1640. It is essential to have almost equal number of Vero cells to get equal rate of viral propagation to study about the comparison of four different batches using two different media and different harvest intervals. The cells were passaged in 25 cm^2 until passage level 158. To get more volume of viral material the cells were sub cultured into 175 cm^2 tissue culture flask in the passage level 159 which are used for virus infection.

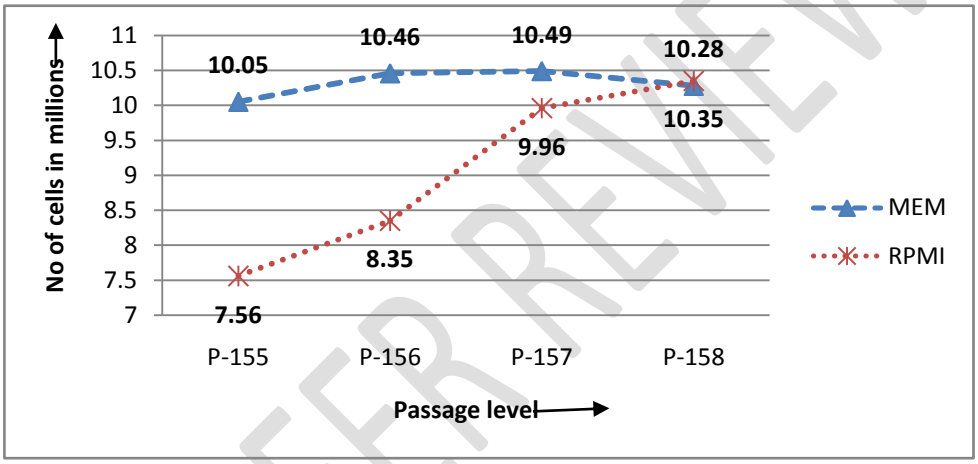


Figure 2: Graphical representation of cell counts obtained in each passage in both media

3.3 Viral Infection and Harvest: The vero cell count of MEM, RPMI 1640 in 175 cm^2 tissue culture flasks were ranged 40.28 x 10^6 cells to 42.06 x 10^6 cells. 0.2 MOI is kept as constant for all 4 batches since to maintain equal status of cell count, virus MOI and media volume of different batches.The tissue culture flasks containing the adhered Vero cells were infected and incubated for 90 minutes to facilitate the attachment of viral particle to the host cells. The optimum number of virus inoculum for infection and availability of cells for virus attachment in initial stage of virus adsorption is considered as important criteria.

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

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

The cytopathic effect on virus infected cells was clearly observed in Vero cells grown in both the media. The microscopic observation of Vero cell on 6th day from viral infection in MEM and RPMI-1640 were shown as *Figure 3.a* and *Figure 3.b* respectively. As per the predefined protocol

the Batch 1,3 was harvested at every 2 days interval and Batch 2,4 were harvested at 3 days interval to study about the correlation between incubation time and virus propagation ratio in terms of viral titer in all 5 viral harvests. The viral harvest was subjected for centrifugation at 600g for 20 minutes under refrigerated condition to remove the cellular debris on the day of each viral harvest. It will eliminate the interference of the cellular debris in the FAT result observation.

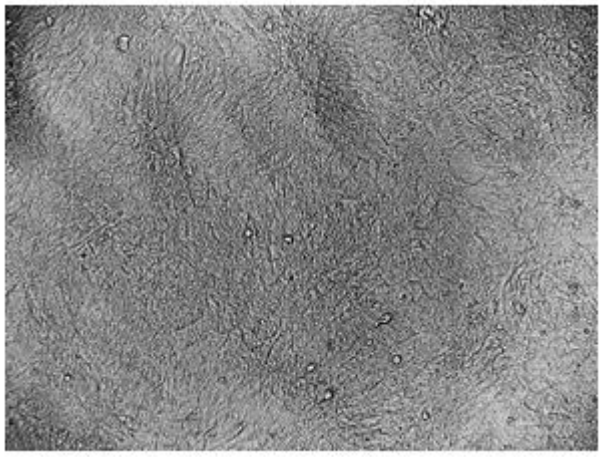


Figure 3.a Microscopic examination of Rabies virus infected vero cells in MEM

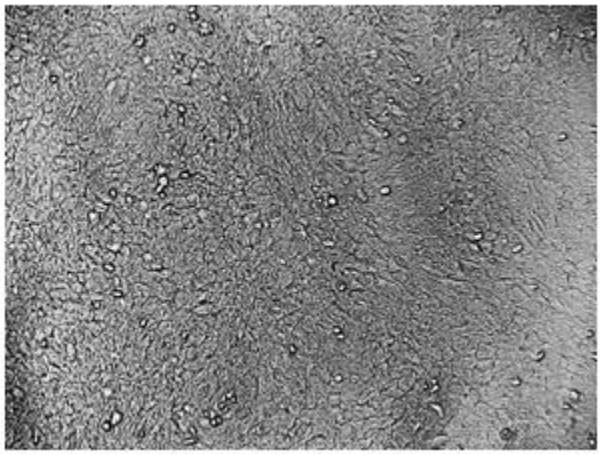


Figure 3.b Microscopic examination of Rabi es virus infected vero cells in RPMI-1640

3.4 Viral titre estimation using FAT test: Fluorescent Antibody Test was used to estimate the viral titer in terms of log value in each of the viral harvest. In this test, the live virus is allowed to infect, propagate in BHK-21 cells and then estimated on the basis of the sample dilution factor up to which the virus can able to infect the BHK 21 cells and the same will be detected by FITC (fluorescein isothiocyanate) antibody conjugate staining procedure.

The fluorescein isothiocyanate as the fluorescent dye was freshly prepared into a conjugate solution along with Anti-Rabies antibody. Under fluorescent microscope, the antigen bound to a fluorescently tagged antibody appears as apple green a fluorescent focus which ensures the presence of propagated virus in respect of particular dilution. The microscopic observation of positive (fluorescent foci) and negative fluorescence in BHK-21 cells under fluorescent microscope was shown in *Figure 4.a & 4.b* respectively. The foci on each well are counted and the infectivity ratio out of 8 wells are used to calculate the viral titre by using Spearman-Karber method.

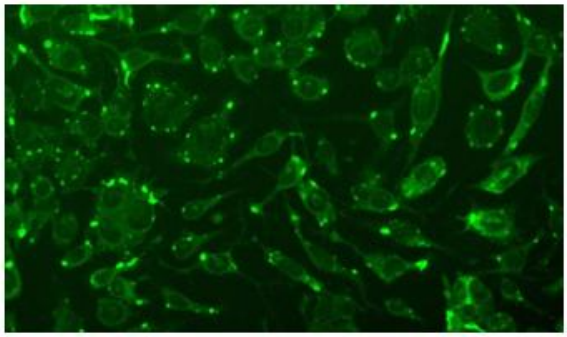


Figure 4.a:Positive fluorescence

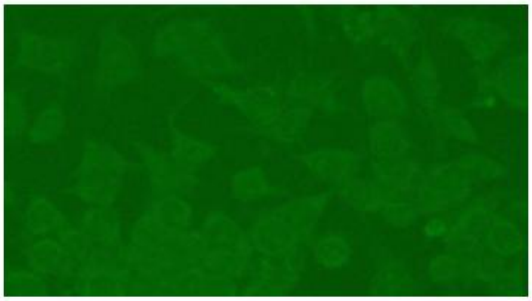


Figure 4.b:Negative fluorescence

The highest viral titre obtained from RPMI-1640 media batch ($10^{-6.125}$/ml) and MEM media batch ($10^{-6.25}$ /ml). Among the different time of viral harvests, Batch 1 & 3 (harvested at 2 days interval), the viral log titre ranged between $10^{-4.375}$ to $10^{-5.875}$and in batch 2 & 4 (harvested at 3 days interval) the viral log titre ranged between $10^{-3.750}$ to $10^{-6.250}$.

3.5 Viral titration*:*

Table *2* Viral Titre values (VH-Viral Harvest)

Batch.No.	Medium & Harvest intervals	Viral Harvests	Virus Titration per ml in log
1.	MEM (harvested at 2 days intervals)	VH-I	$10^{-5.019}$
		VH-II	$10^{-5.875}$
		VH-III	$10^{-5.625}$
		VH-IV	$10^{-5.125}$
		VH-V	$10^{-4.625}$
2.	MEM (harvested at 3 days intervals)	VH-I	$10^{-5.875}$
		VH-II	$10^{-6.25}$
		VH-III	$10^{-6.125}$
		VH-IV	$10^{-5.375}$
		VH-V	$10^{-3.75}$
3.	RPMI 1640 (harvested at 2 days intervals)	VH-I	$10^{-5.186}$
		VH-II	$10^{-5.375}$
		VH-III	$10^{-5.75}$
		VH-IV	$10^{-5.125}$
		VH-V	$10^{-4.375}$
4.	RPMI 1640 (harvested at 3 days intervals)	VH-I	$10^{-5.375}$
		VH-II	$10^{-6.125}$
		VH-III	$10^{-5.875}$
		VH-IV	$10^{-5.125}$

		VH-V	$10^{-3.875}$

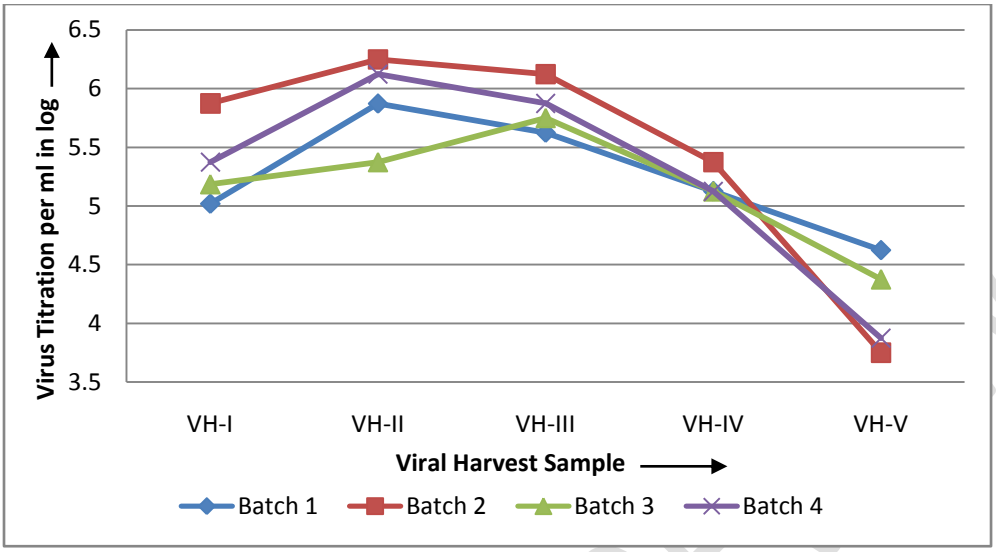


Figure 5: Graphical representation of viral titration of all the samples obtained using FAT test

Conclusion:

Both MEM and RPMI 1640 tissue culture media equally supported the growth of vero cells and there is no much difference in rabies viral titre. It is observed that the two-day viral harvesting system has viral titre lower than the 3 days harvest upto 4th harvest and higher in 5th harvest. Both the media behaved in the similar pattern. The viral propagation needs some minimal time to reach the higher log value since the 2 days interval harvest show lower log value than 3 days viral harvest. Based on this observation, it is concluded that the optimal time of harvest at 3 days interval with any one of these tissue culture medium . It is a preliminary study of propagation of Rabies virus to get higher yield in cell culture bottles and it can be extended with multiple stack cell factories to get more viral titre.

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