Studies on the potency of oral polio vaccine using RD cell line and evaluation of growth using different serum concentration and volume of media

V. S. Tom Robins¹, R. Dhivyā¹*, R. Sri Ramji Kumar¹, T. Lalitha² and K. N. Venkataramana²

¹Kongunadu Arts and Science College, Coimbatore, VIT University, Vellore, KS Rangasamy College of Technology, Thiruchengode, India.
²National Polio Surveillance Lab, Pasteur Institute of India, Coonoor, India.

Accepted 27 July, 2009

Oral polio vaccine (OPV) proved to be superior in administration eliminating the need of sterile syringes and making the vaccine more suitable for mass vaccination campaigns. Poliovirus is heat sensitive in nature, and thus OPV is stored at low temperature (frozen). The growth medium containing varying concentration of serum such as 6, 8, 10, 12, 14% were prepared. 10 ml of the above mentioned growth media containing different concentration of serum were added to different culture bottles. The culture flasks containing different volumes of growth medium with 10% serum concentration such as 8, 9, 10, 11 and 12 ml were added to a series of culture flasks. All the culture flasks were inoculated with the RD cells (10,000 cells/culture flask) and kept at 37°C. The most favoured serum concentration and volume for the growth of RD cells was found and used for testing the potency of vaccine. Vaccines from two manufacturers were kept at three different temperatures, 2-8 ± 0.5°C (refrigerator), 26 ± 0.5°C and 37 ± 0.5°C (Incubator). Cytopathic viruses were titrated by the determination of a tissue culture infectious dose ₅₀ (TCID₅₀), vaccine dilutions were seeded in replicate onto cells in multiwell plates (usually 96 wells). After a suitable incubation period, wells were examined microscopically and scored as infected or not infected. The potency of vaccines was tested using the Karber’s Formula.

Key words: Oral polio vaccine, rhabdomyosarcoma, thermostability, potency.

INTRODUCTION

Poliomyelitis, often called polio or infantile paralysis, is an acute viral infectious disease spread from person to person, primarily via the faecal-oral route. Although effective poliovirus vaccines have been available for over a quarter of a century, paralytic poliomyelitis remains a serious health risk in many countries. In developing countries the incidence of the disease remains high. Even though concerted vaccination campaigns have improved the situation in certain regions, more urgent health priorities or difficulties arising from poor economies or political uncertainty, have meant that vaccination against poliomyelitis worldwide has not been sufficiently comprehensive to have had a major impact on the total global incidence of the disease. Even in developed countries, poliomyelitis, although greatly diminished in incidence, has not disappeared. Continuing circulation of wild-type strains and regular importation of virus from the third world necessitates continued vigilance in maintaining high levels of immunity. Although developed countries generally experienced a dramatic decrease in poliomyelitis after vaccination was introduced, those countries using the live-attenuated vaccines developed by Albert Bruce Sabin have noted a residual low level of approximately 0.02 - 0.2 cases/million of population/years. Evidence has accumulated that this persistent low level of disease may be caused by the vaccines themselves, especially those of serotypes 2 and 3.

Polioviruses are members of the Enterovirus genus of the family Picornaviridae. The three distinct serotypes 1, 2 and 3 cause identical disease and are very similar in structure and composition. In each case the 27 nm
diameter icosahedral capsid consists of 60 copies each of four virus polypeptides, VP1-VP4, surrounding a single stranded, messenger-sense RNA genome of approximately 7450 nucleotides (Almond et al., 1984).

Most vaccines, live vaccines particularly, are temperature sensitive. To ensure an adequate shelf life, they should be kept at a low temperature. Consequently, their delivery requires the provision of a cold chain. The least stable of the common childhood vaccines is the live attenuated polio vaccine (OPV). It is a non-freeze-dried vaccine and is composed of a single attenuated strain of each of the three serotypes (Rombaut et al., 1996).

Thermostability requirements were defined by the WHO that, fall in titre value for the three vaccine strains after exposure to 37°C for two days should be less than 0.5 log10 of titre. In addition current regulations require that, for maintenance of potency, the vaccine must be stored and shipped frozen and that, after thawing, it must be held in the refrigerator at no more than 10°C for a period not to exceed 30 days, after which time it must be discarded (Rombaut et al., 1996), the ideal preparation should deliver the antigen in such a way that a long lasting effect is achieved and provides protection. The studies have been undertaken to study the stability of OPV at different temperatures and the effect of varying serum concentrations and volume on the medium used to grow the RD cells that are utilized for the potency test and to measure the tolerance of the OPV to higher temperature, thus creating an awareness for the importance of cold chain maintenance.

MATERIALS AND METHODS

Revival of cells

The liquid nitrogen storage register was checked and the cell seeds to be revived were selected. The vials were transferred immediately to a beaker of water maintained at 36°C. The contents were thawed completely, the vials were wiped from outside with alcohol to reduce bacterial contamination. 1 ml of 10% Growth Medium was added drop by drop and shook gently after each addition. The content was transferred into 10ml of the growth medium in a culture bottle drop by drop. The culture bottle was incubated at 36°C for 5 - 6 h till the cells adhered. If the cells had adhered during this period it was discarded (Rombaut et al., 1996), the ideal preparation should deliver the antigen in such a way that a long lasting effect is achieved and provides protection. The studies have been undertaken to study the stability of OPV at different temperatures and the effect of varying serum concentrations and volume on the medium used to grow the RD cells that are utilized for the potency test and to measure the tolerance of the OPV to higher temperature, thus creating an awareness for the importance of cold chain maintenance.

Cell splitting

The culture flasks (25 cm²) were examined for confluent monolayer of RD cell lines for Quality and absence of contamination. The growth medium was decanted from the cell culture flasks and gently washed the confluent monolayer with 3 ml incomplete PBS twice. 3 ml of trypsin was added to the monolayer and dispersed evenly. Trypsin was decanted off the flasks and left until streaks appeared at the edges. The flask was tapped few times against the hand to detach all the cells. The cells were re-suspended in 5 ml growth medium, which neutralize the action of trypsin. The suspension was gently aspirated few times with a pipette to break up cell clumps.

The culture bottle was performed and diluted in 10% growth medium. After seeding of cells, the fresh culture flasks were capped tightly and placed in a 36°C incubator. The growth medium (10%) in the culture flask was changed to maintenance medium (2%) after a monolayer has formed on the fifth day, if required.

Cell counting

The monolayer cell culture was trypsinized. To each well of the 96 well microtitre plates 0.1ml of the cell suspension containing approximately 10,000 cells were added. The plates were then incubated at 37°C for one day in 5% CO₂ atmosphere. The medium from all the wells were removed by flicking. The trypsin was added to all the wells (0.1 ml/well). The plates were incubated at 37°C for 3 - 5 min, and then 1 ml of medium was added to all the wells. The wells were dispersed and cell suspension was pooled from the wells to eppendorf tubes. Equal amount of tryphan blue dye was added and cell count was done using Haemocytometer. Discarded and re-suspended the original cell suspension if cell clumping was observed. The mean count of the cells was calculated.

The cell concentration/ml was calculated using the following formula

\[ C1 = m x tb x 10^4 \]

Where m = Mean of the cell count of the 4 corners, \( tb \) = correction to the tryphan blue dilution, and \( C1 \) = Initial cell concentration/ml. Depending on the count, the cells were seeded into bottles and incubated at 36°C.

Serum concentration

The normal serum concentration in GM is 10%. The effects of varying serum concentrations of 6, 8, 10, 12 and 14% on the growth of RD cells used for the potency test were studied. The best concentration of the serum in which the maximum cell growth achieved was selected to be the optimum concentration of serum.

Volume of media

The Rhabdomyosarcoma cells used for the potency test were grown in different volumes of growth medium containing 10% serum, different volumes of growth media such as 8, 9, 10, 11 and 12 ml were selected for the study. The best volume of serum that helps in the growth of RD cells was found out by the study.

Estimation of potency for vaccines kept at 2-8 ± 0.5°C, 26 ± 0.5°C and 37 ± 0.5°C

RD culture bottle with confluent monolayer was selected. Cells not more than 2 days old were taken. The medium was discarded. The cells were trypsinized, counted and seeded accordingly. Three different sterile microtitre plates were chosen for the titration of vaccines kept at 2-8 ± 0.5°C (refrigerator), 26 ± 0.5°C and 37 ± 0.5°C (incubator) respectively. The vaccine samples were serially diluted. 100 µl of growth medium containing 10,000 cells were added to all the wells. 100 µl of growth medium was discarded. The control wells was incubated at 37°C for one week. This reading of potency was observed. The mean count of the cells was calculated.
Table 1. Growth of RD cells vs. serum concentration of GM.

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Concentrations of serum</th>
<th>Cell count millions/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6%</td>
<td>3.843</td>
</tr>
<tr>
<td>2</td>
<td>8%</td>
<td>4.623</td>
</tr>
<tr>
<td>3</td>
<td>10%</td>
<td>4.80</td>
</tr>
<tr>
<td>4</td>
<td>12%</td>
<td>5.112</td>
</tr>
<tr>
<td>5</td>
<td>14%</td>
<td>6.540</td>
</tr>
</tbody>
</table>

*The cell count is expressed in millions/ml.

Figure 1. Growth of RD cells vs. serum concentration of GM.

The effect of varying serum concentration in the growth media was studied with respect to the growth of RD cell line. The cell growth was known to increase with serum concentration. The values for cell count are presented in Table 1 and Figure 1.

Different volumes of the media were studied with respect to their effect on RD cell growth after observing for three days. Complete monolayer was formed on the day of observation. The values for cell count are presented in Table 2 and Figure 2.

Thus the growth medium containing 10% serum concentration and 10 ml volume of media was selected for the growth of RD cells as the monolayer formation was convenient for the study to be carried on testing the potency of vaccine.

The potency of the given vaccine samples A and B were tested by incubating at three different temperatures. The samples were kept at 26 ± 0.5°C, 2-8 ± 0.5°C, and 37 ± 0.5°C for various periods of incubation.

Infectivity titre method was employed to find out the drop in potency of the samples, the potency drop was checked periodically in specific intervals, the culture which was incubated with vaccines would show cytopathic effect after a certain period of incubation, the cultures showing cytopathic effect was counted and potency drop was calculated using Karber’s formula.

Sample A

The initial titre was 6.93 log TCID_{50} and the samples which were exposed to 2-8 ± 0.5°C started declining after
Table 2. Growth of RD cells at different volumes of media.

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Volume of Media</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>Cell Count*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 ml</td>
<td>7.8</td>
<td>7.06</td>
<td>2.050</td>
</tr>
<tr>
<td>2</td>
<td>9 ml</td>
<td>7.8</td>
<td>7.16</td>
<td>2.600</td>
</tr>
<tr>
<td>3</td>
<td>10 ml</td>
<td>7.8</td>
<td>7.60</td>
<td>4.300</td>
</tr>
<tr>
<td>4</td>
<td>11 ml</td>
<td>7.8</td>
<td>7.90</td>
<td>5.100</td>
</tr>
<tr>
<td>5</td>
<td>12 ml</td>
<td>7.8</td>
<td>7.70</td>
<td>6.450</td>
</tr>
</tbody>
</table>

*The cell count is expressed in millions/ml.

Figure 2. Growth of RD cells at different volumes of media.

Table 3. Virus titre at 26 ± 0.5°C.

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Temperature</th>
<th>Days</th>
<th>Sample A*</th>
<th>Sample B*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-20°C</td>
<td>0th</td>
<td>-6.82</td>
<td>-6.88</td>
</tr>
<tr>
<td>2</td>
<td>+26°C</td>
<td>7th</td>
<td>-5.90</td>
<td>-6.01</td>
</tr>
<tr>
<td>3</td>
<td>+26°C</td>
<td>14th</td>
<td>-5.1</td>
<td>-5.3</td>
</tr>
<tr>
<td>4</td>
<td>+26°C</td>
<td>21st</td>
<td>-4.5</td>
<td>-4.6</td>
</tr>
<tr>
<td>5</td>
<td>+26°C</td>
<td>28th</td>
<td>-4.0</td>
<td>-4.11</td>
</tr>
<tr>
<td>6</td>
<td>+26°C</td>
<td>35th</td>
<td>-3.2</td>
<td>-3.4</td>
</tr>
</tbody>
</table>

*The values of sample A, sample B are expressed as the negative log of TCID\(_{50}\) of the vaccine exposed to the respective temperature.

seven days. A drop of 0.33 log TCID\(_{50}\) was noticed from 7\(^{th}\) day and further drop of 0.53 log TCID\(_{50}\) was noticed on the 14\(^{th}\) day. Since the initial vaccine titre was more than the requirement, the vaccine was able to pass the minimum requirement in spite of 0.5 log drop. But at 21\(^{st}\), 28\(^{th}\), 35\(^{th}\) day, the drop was more than the 0.5 log. Hence the vaccine failed to comply with the minimum requirement. In the case of the samples being exposed to 26 ± 0.5°C, the drop in titre was noticed on the 7\(^{th}\) day and the titre was below the minimum requirement. In the case of the samples being exposed to 37 ± 0.5°C, the drop in titre was significant within 24 h and by the second day the virus titre was below the minimum requirement. The values are presented in the Tables 3, 4 and 5, and
Table 4. Virus titre at 2-8 ± 0.5°C.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Temperature</th>
<th>Days</th>
<th>Sample A*</th>
<th>Sample B*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>-20°C</td>
<td>0th</td>
<td>-6.93</td>
<td>-6.85</td>
</tr>
<tr>
<td>2.</td>
<td>2-8°C</td>
<td>7th</td>
<td>-6.60</td>
<td>-6.5</td>
</tr>
<tr>
<td>3.</td>
<td>2-8°C</td>
<td>14th</td>
<td>-6.43</td>
<td>-6.33</td>
</tr>
<tr>
<td>4.</td>
<td>2-8°C</td>
<td>21st</td>
<td>-6.1</td>
<td>-6.17</td>
</tr>
<tr>
<td>5.</td>
<td>2-8°C</td>
<td>28th</td>
<td>-5.8</td>
<td>-5.9</td>
</tr>
<tr>
<td>6.</td>
<td>2-8°C</td>
<td>35th</td>
<td>-5.4</td>
<td>-5.5</td>
</tr>
</tbody>
</table>

*The values of sample A, sample B are expressed as the negative log of TCID$_{50}$ of the vaccine exposed to the respective temperature.

Table 5. Virus titre at 37 ± 0.5°C.

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Temperature</th>
<th>Days</th>
<th>Sample A*</th>
<th>Sample B*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>-20°C</td>
<td>0th</td>
<td>-6.63</td>
<td>-6.69</td>
</tr>
<tr>
<td>2.</td>
<td>+37°C</td>
<td>1st</td>
<td>-6.43</td>
<td>-5.93</td>
</tr>
<tr>
<td>3.</td>
<td>+37°C</td>
<td>2nd</td>
<td>-5.93</td>
<td>-5.75</td>
</tr>
<tr>
<td>4.</td>
<td>+37°C</td>
<td>3rd</td>
<td>-5.62</td>
<td>-5.50</td>
</tr>
<tr>
<td>5.</td>
<td>+37°C</td>
<td>4th</td>
<td>-5.50</td>
<td>-5.37</td>
</tr>
<tr>
<td>6.</td>
<td>+37°C</td>
<td>5th</td>
<td>-5.36</td>
<td>-5.21</td>
</tr>
</tbody>
</table>

*The values of sample A, sample B are expressed as the negative log of TCID$_{50}$ of the vaccine exposed to the respective temperature.

Figures 3, 4 and 5.

**Sample B**

The initial titre was 6.85 log TCID$_{50}$ and the samples which were exposed to 2-8 ± 0.5°C started declining after 7 days. A drop of 0.35 log TCID$_{50}$ was noticed from 7th day and further drop of 0.52 log TCID$_{50}$ was noticed on the 14th day. Since the initial vaccine titre was more than the requirement, the vaccine was able to pass the minimum requirement in spite of 0.5 log drop. In the case of
the samples being exposed to 26 ± 0.5°C, the drop in titre was noticed on the 7th day, a drop of 0.87 log TCID$_{50}$ was noticed. Since the vaccine titre was not more than the minimum requirement, the vaccine was not able to pass the minimum requirement in spite of 0.5 log drop. In the case of the samples being exposed to 37 ± 0.5°C, the drop in titre was noticed within 24 h and there was a drop of 0.86 log TCID$_{50}$ on the seventh day. Further drop of 1.32 log TCID$_{50}$ was noticed at 4th day. The vaccine titre was below the minimum requirement. The values are presented in the Tables 3, 4 and 5, Figures: 3, 4 and 5.

**DISCUSSION**

**Growth of RD cells vs. serum concentration of GM**

8% serum concentration supported the growth of RD
cells in the same way as that of 10 and 6% serum concentra-
tion showed a poor cell count. The cell growth was
known to increase with serum concentration, but in the
case of 12 and 14%, confluent monolayer was formed on
the second day itself. The cells detached from the
surface on the third day that resulted in decreased avail-
ability of cells for the potency test. Therefore 10% serum
concentration was selected for the growth of RD cells that
were used for testing the potency of vaccine.

Growth of RD cells at different volumes of media

The normal volume of media used for the growth of RD
cells is 10 ml. Varying volumes of the media were studied
with respect to their effect on RD cell growth after
observing for three days. 10, 11 and 12 ml, of the growth
media imparted effective growth on RD cells as
evidenced by high cell count, whereas 8 and 9 ml of the
media showed poor cell count when compared with other
volumes of media under study. 10 ml of the growth me-
dium was selected for the growth of RD cells as the
amount was affordable, monolayer formation and cell
count was sufficient to carry out the potency testing of
vaccine.

Potency drop at various temperatures

The fall in infectivity is due to the hydrolysis of the geno-
ic RNA by the RNA polymerase within the virus particle.
This can be greatly inhibited by the suppression of the
enzymatic activity which can be achieved by reducing the
pH below 7 (Newman et al., 1995).

Potency drop at 26 ± 0.5°C

At 26 ± 0.5°C the vaccine potency was found out to be -6.82 for Sample A and -6.88 for Sample B and the
potency drops according to the duration of exposure to
the temperature. After 7 days of incubation the potency
dropped to a value of -5.9 and -6.01 for Sample A and
Sample B respectively. At 22°C the average loss in virus
titre after 21 days was about 1.50 (Sokhey et al., 1988).
In a study conducted previously whereas in our study the
average loss in virus titre after 21 days at 26 ± 0.5°C was
about 2.30. Thus proving that the potency drops as the
temperature of incubation of the vaccine increases.

Potency drop at 2-8 ± 0.5°C

The vaccine that was incubated in 2-8 ± 0.5°C shows a
lesser amount of potency drop like that of another study
where not much loss in virus titre in three samples stored
at 4 - 8°C for a year was observed temperatures (Sokhey
et al., 1988). The average loss in virus titre in a year (log
TCID50) was 0.65 at 4 - 8°C when various samples were
stored at these whereas in our study 0.5 log drop was
observed at the 14th day of incubation itself.

Potency drop at 37 ± 0.5°C

The drop in potency of the vaccine kept at 37 ± 0.5°C
was rapid. The vaccine showed a good margin of potency
loss after 5 days of incubation. The initial potency has
been calculated and it is -6.63 and -6.69 for the Samples
A and B respectively. After the incubation of 5 days in 37 ± 0.5°C potency was found to be -5.36 and -5.21 for
Sample A and Sample B respectively in the same way as
that of a study conducted earlier where the samples
exposed to 36°C for 21 days showed almost complete
loss in virus titre (Sokhey et al., 1988).

Conclusion

The expanded programme of immunization (EPI) was
launched globally in 1978 and it targeted on six childhood
diseases, which includes poliomyelitis. In India universal
immunization programme was launched in 1985, prior to
1997, surveillance for polio was directed at finding clinical
polio cases by passive reporting from health facilities.
The number of polio cases in India has dropped from an
estimated 32,630 in 1995, before the pulse polio
programme began to just 268 in 2001. The number of
polio cases associated with isolation of wild poliovirus
decreased from 1934 in 1998, 1126 in 1999, 265 in 2000
and 268 in 2001. Polio has disappeared from large parts
of country. By the end of 2001 most of India was either
polio free or in a good position to achieve that status
shortly thereafter (AFP alert NPSP, 2001).

Studies have been undertaken as poliovirus is
thermolabile which requires continuous monitoring of cold
chain. Exposure of these viruses to higher temperature
destroys them rapidly and makes the vaccine unusable.
Since there is a close relationship between storage
temperature and poliovirus survival, manufacturers re-
commend expiry dates for OPV according to the tempera-
ture at which it is kept. Many give two figures: (1) Up to
two years if the vaccine is stored in a deep freezer at or
below -20°C; and (2) for six months if it is stored in a
refrigerator at 0-8°C. One manufacturer stated that
magnesium chloride-stabilized vaccine maintains ade-
quate immunogenicity for 12 months when kept in a
refrigerator at 2 - 8°C, for three weeks at 25°C, and for
three days at 37°C.

Our study clearly showed that the live attenuated polio
vaccine does not retain its potency if the cold chain main-
tenance is not proper. Whenever the live polio vaccine is
exposed to a temperature greater than freezing tempe-
Rature the potency unit drops. The drop in potency of
vaccine seems to be directly proportional to the increase in temperature. The samples kept at -20°C retained full potency for the entire storage period and was used as control in this present study. The potency drop at 2 - 8°C after 35\textsuperscript{th} day was 22.07% for Sample A and 19.70% for Sample B, the drop in potency for the vaccine kept at 26°C after the 35\textsuperscript{th} day was 53.07% for Sample A and 50.14% for Sample B and the potency of vaccine decreased significantly as it was exposed to 37°C, the drop in potency for Sample A was 19.15% after the 5\textsuperscript{th} day and for Sample B 22.12%. Previous studies show that Oral poliomyelitis vaccines may lose 4 - 13% of their activity per day at 25°C, 11 - 21% per day at 31°C and 26 - 34% per day at 37°C. Poliomyelitis vaccines retained minimum potency for three days at 37°C and 14 to 21 days at 25 - 28°C.

The RD cells which were used for the study showed maximum growth in 14% fetal calf serum and when the serum concentration is fixed to 10%, it showed the maximum growth in the culture with 12 ml.

REFERENCES