Comparison of two colorimetric assays to determine viral infectivity in micro culture virus titration

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Efficacy of two colorimetric assays, viz. MTT (3-(4,5-dimethylthiazol-2-(yl-2,5-diphenyl tetrazolium bromide) and neutral red (NR) assays, performed by integrating them to micro culture virus titration (MCVT), was compared with the conventional MCVT method in terms of percentages of infectivity and 50% infectivity end points by employing Polio virus type-3 and Dengue virus type 4 as the candidate viruses. The results suggested that MTT assay has an edge over NR assay as well as conventional MCVT method. For the first time, NR assay has been successfully employed for the determination of virus infectivity titre.

Measurement of infectivity potential of a given virus is of paramount importance for accomplishing immunological studies, vaccine preparation etc. Cytopathic viruses have been conventionally quantitated by plaque assay and titrated for determination of 50% infectivity end points. However, inherent problems e.g. manual counting of plaques apart from variation in plaque size and monitoring of cytopathic effects (CPE) for titration of infectivity render these tests vulnerable to erroneous results.

Colorimetric assays, on the other hand, quantify cell viability through enzyme mediated biochemical reactions owing to ingress of certain dyes inside the living cells. Mosmann and Borenfreund and Puerer first advocated the application of tetrazolium (MTT) and neutral red (NR) assay respectively, to quantitate cell viability and eventually the cytotoxicity to cells in vitro.

MTT assay, also known as tetrazolium assay, has been exploited extensively to reveal the protective efficacy of therapeutic agents and plant extracts against cancer, human immuno deficiency virus (HIV-1) and herpes simplex virus and determination of neutralizing antibody levels to HIV and respiratory syncytial virus. MTT assay using micro culture virus titration (MCVT) was applied for the determination of infectivity titres of influenza viruses and was found to be compatible with the well established procedure of egg infectivity assay. Unlike MTT, neutral red dye uptake assay has not been substantially exploited in virological research. NR dye assay was earlier performed for the study of antiviral efficacy of basil leaves extract against Polio virus type 3.

In the present study comparative efficacy of MTT and NR assay systems vis-a-vis conventional method of determining infectivity end point in MCVT employing polio virus type-3 (Polio-3) dengue virus type - 4 (DEN - 4) as candidate viruses have been reported.

Materials and Methods

Cells—Human foetal lung epithelial cell line (L-132) and African green monkey kidney cell line (Vero) obtained from National Centre for Cell Science (Pune, India) were maintained in our laboratory by regular sub passaging using Eagle's minimum essential medium (EMEM) supplemented with 10% neonatal calf serum (NCS-Sigma, USA). L-132 and vero cell monolayers, between passage (P) levels 120-130 and P40-50 respectively, were utilized in the present study.

Candidate viruses—Polio-3 and DEN-4 viruses were obtained from National Institute of Health (Bethesda, USA) and National Institute of Virology (Pune, India) respectively. They were maintained in our laboratory through serial passages, as per the standard procedure. Eventually both the viruses propagated at P10 level were employed for the present study.

Antisera—Monospecific monkey antiserum against Polio-3 was obtained from Christian Medical College
(Vellore, India) whereas antiserum to DEN-4 in mice was raised by us. These were subjected to checker board titration in indirect ELISA before use.

**Enzyme linked immunosorbent assay (ELISA)—** Presence of the virus was confirmed by indirect ELISA based on the basic principles described earlier\(^3\) incorporating certain modifications. The cells were fixed with 10% phosphate buffer saline (PBS) formalin on the attainment of 70-80% cytopathic effects (CPE), blocking the wells with 5% egg albumin (EA), incubating at 37°C for 2 hrs and then addition of antiserum of the respective viruses. Goat anti human and rabbit anti mouse IgG horse radish peroxidase conjugates were added in the respective ELISA plates. After interaction with ortho phenylene diamine+H\(_2\)O\(_2\) the reaction in the wells were stopped with 2N H\(_2\)SO\(_4\) and the optical densities recorded at 490 nm absorbance.

**Conventional MCI VT—** Determination of tissue culture infective dose 50% (TCID\(_{50}\)) end point by conventional method was performed as per standard procedure\(^{11}\). Freshly trypsinized L-132 cells containing 5 \(\times\) 10\(^4\) cells/ml, suspended in EMEM with 10% neonatal calf serum (NCS) were seeded (100 µl/well) in MT plate and incubated at 37°C in 5% CO\(_2\) tension. Serial 10 fold dilutions of Polio-3 and DEN-4 (10\(^{-1}\) to 10\(^8\)) were prepared alongside and 100 µl/well of each dilution was added in a set of 8 wells onto confluent cell monolayer, earmarking two rows of healthy cells (untreated) as control. Infected cells were monitored each day for the development of viral induced CPE which occurred maximally by day 3 post infection (PI) in case of Polio-3 and 4 to 5 days PI in DEN-4. Tissue culture infective dose (TCID\(_{50}\)) was calculated according to the formula of Reed and Muench\(^{11}\).

**Colorimetric assays—** MTT and NR dye assays performed in MCVT were identical in procedural details up to the development of maximal CPE induced by different concentrations of the aforesaid viruses. Additional protocols with regard to the two assay systems were as follows:

(i) **MTT assay:** MTT assay for virus infectivity was performed in accordance with the method of Mosmann\(^4\). The cells were washed with PBS on the maximal production of CPE and replenished with 100 µl/well of EMEM without phenol red. Tetrazolium dye (Sigma, USA) dissolved in PBS (5mg/ml) was added to each well (10 µl/well) and incubated at 37°C in a CO\(_2\) incubator for 3 hr. Thereafter, 15 µl acid propanol (0.04 N HCl in isopropanol) was added to each well and mixed thoroughly. Optical densities (OD) were recorded at 570 nm absorbance in an ELISA Reader (Dynatech, USA).

(ii) **Neutral red (NR) assay:** This assay was accomplished according to the procedure of Borenfreund and Puerner\(^5\) with certain modifications. Briefly, the cells were washed with PBS on the production of maximal CPE, fixed with 4.5% gluteraldehyde solution, kept at room temperature for 45 min, further washed with PBS, stained with 0.2% NR dye aqueous solution and incubated for 1 hr at room temperature. After thorough washing the stained cells were subjected to acid alcohol lysis by adding 100 µl of 0.5% acetic acid (V/V) in 50% ethanol to each well. After 10 min of incubation, OD values were recorded as indicated above.

**Computation of TCID\(_{50}\) in colorimetric assays—** The infectivity end point (TCID\(_{50}\)/ml) of a given virus was calculated by determining initially the cut off values. Cut off values were calculated according to Levi et al\(^{1}\). Initially the OD values of the blanks comprising mixtures used for the liberation of the end products of the dyes (MTT and NR) were monitored and deducted from the experimental OD readings. Thereafter the cut off values were determined as follows:

Mean OD value of untreated control wells - 2 \(\times\) standard deviation (SD)

**Interpretation:**
- OD < cut off value indicated positivity
- OD > cut off value indicated negativity

Subsequently, the percentages of infectivity for each dilution was calculated by taking proportion of positivity of 8 wells per dilution. Finally, the 50% infectivity end point (log 10 TCID\(_{50}\)/ml) was calculated in accordance with Reed and Muench formula\(^{11}\).

**Results**

Colorimetric assays offer measurement oriented results which have been described as the mean of minimum three replicates. Initially the presence of viruses was confirmed by an indirect ELISA wherein fixed cells exhibited positive reactivity in infected wells as compared to relative negative reactivity in control wells. An elevated 50% infectivity end point of 7.0 (log 10 TCID\(_{50}\)) was observed in MTT assay as compared to 6.5 in NR and MCVT assays in respect
Table 1—Comparative 50% infectivity end point (TCID<sub>50</sub>) by colorimetric assays (MTT and NR) and the conventional MCVT

<table>
<thead>
<tr>
<th>Assay procedure</th>
<th>Log&lt;sub&gt;10&lt;/sub&gt; TCID&lt;sub&gt;50&lt;/sub&gt;/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polio-3</td>
<td></td>
</tr>
<tr>
<td>MTT</td>
<td>7.0</td>
</tr>
<tr>
<td>NR</td>
<td>6.5</td>
</tr>
<tr>
<td>MCVT</td>
<td>6.5</td>
</tr>
<tr>
<td>DEN-4</td>
<td></td>
</tr>
<tr>
<td>MTT</td>
<td>4.0</td>
</tr>
<tr>
<td>NR</td>
<td>3.66</td>
</tr>
<tr>
<td>MCVT</td>
<td>3.00</td>
</tr>
</tbody>
</table>

Log<sub>10</sub> TCID<sub>50</sub> was determined after calculating the proportions of positivity of 8 wells/dilution and the cut off values thus obtained were designated Positive = > cut off value, Negative = < cut off value.

of Polio-3 (Table 1). On the other hand, DEN-4 exhibited relatively reduced infectivity titres, though higher by MTT assay (4.0) than NR and MCVT assays which in turn revealed 3.66 and 3.0 infectivity end points respectively. Polio-3 virus revealed almost identical percentages of infectivity in conventional MCVT and NR assays except at 10<sup>-3</sup> dilution of the virus wherein 100% infectivity was detected in contrast to 87.5% by MCVT (Fig. 1). MTT assay invariably revealed an edge over NR and MCVT. Even at the lowest dilution of 10<sup>-4</sup>, 12.5% infectivity was detected in MTT assay whereas no infectivity was evident by the other two tests (Fig. 1a). In DEN-4 also the results indicated an invariably higher percentages of infectivity by MTT assay. A comparison between NR and MCVT revealed that NR exhibited elevated infectivity percentages as compared to MCVT (Fig. 1b).

The quantification of cell viability (expressed in terms of average of OD values) vis-a-vis corresponding virus dilutions of Polio-3 and DEN-4 have been depicted in Fig. 2 (a and b). A dose dependent response is clearly evident as indicated by the increase in cell viability with proportionate decrease in the concentration of both the viruses.

**Discussion**

In colorimetric assays MTT and NR dyes get converted into coloured products in response to the biochemical reaction in the living cells. In turn these products can be measured in terms of optical densities. MTT—a Tetrazolium salt is a yellow coloured dye (3-(4,5 dimethylthiazol 2-yl) 2,5 diphenyl tetrazolium bromide) which gets cleaved by mitochondrial succinate dehydrogenase enzyme into a blue coloured formazan in active cells. This product does not form crystals when interacted with isopropyl alcohol and thus can be accurately measured<sup>3</sup>. On the other hand, neutral red—a weak cationic dye gets accumulated in the lysosomes of the living cells that can then be extracted by acid-alcohol lysis<sup>4</sup>.

The conventional MCVT is a quantal method which depends on the visual observation and subjective
scoring of CPE, elicited in response to different concentrations of the viruses. Colorimetric assays on the contrary are independent of CPE manifestations, reveal the extent of cell viability quantitatively and thereby indirectly denote the virus infectivity. MCVT when coupled with colorimetric procedure ensures reproducibility, consistency and ease of performance. This apart, in MTT assay there is no need to harvest the viable cells wherein the cell viability can be directly measured by a spectrophotometer. In NR assay however, the extraction of dye is an imperative step to record the corresponding OD values. MTT dye has been reported to be applied in various assay systems where quantification of living cells can indirectly denote the state of the cytotoxic cells viz. quantification of cells in myotoxic assay of snake venoms, assay of interleukin-2 by determining helper T cell population, response of chemotherapy in leukemia etc. NR dye was however, exploited only as an indicator of cytopathogenicity of viruses in an interferon assay. However, no direct reports on the application of NR dye for determining the virus infectivity end point are available. Therefore to our knowledge this study may be the first attempt of titrating virus infectivity by employing the NR dye.

In the present study, a titre of 7.0 TCID₅₀/ml was detected in respect of Polio-3 by MTT assay whereas a lower titre of 6.5 was exhibited by both NR and MCVT assays. In comparison, DEN-1 showed relatively decreased titres of 4.0, 3.6 and 3.0 TCID₅₀/ml by MCVT, NR and MCVT respectively (Table 1). Percentages of infectivity (Fig. 1) further substantiated the relative efficacy of MTT assay to quantify the extent of CPE elicited by different dilutions of both the viruses. Dose dependent response as exhibited by colorimetric assays accurately measured the extent of cell damage by different concentrations of the two candidate viruses. The lower OD values in MTT assay, as compared to NR assay at a given concentration of the virus, denote higher sensitivity of MTT which in turn represents the mitochondrial status of the cells (Fig. 2). Though a recent report suggested a good correlation between MTT and NR assays with regard to evaluation of cytotoxicity, the present results revealed an edge of MTT assay system over both NR and the conventional MCVT in respect of infectivity end point determination of the viruses.

The present study conclusively suggests that the precision and ease of performance of colorimetric assays for the determination of the infectivity titres of cytopathic viruses offer a better alternative to the existing techniques for infectivity determination. Between the two colorimetric assay procedures, MTT is relatively more sensitive and less cumbersome than NR assay.

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References