<table>
<thead>
<tr>
<th>項目</th>
<th>内容</th>
</tr>
</thead>
<tbody>
<tr>
<td>题目</td>
<td>デングウイルスの迅速定量法の感受性について</td>
</tr>
<tr>
<td>作者</td>
<td>牧野 芳大、三舟 求真人</td>
</tr>
<tr>
<td>雑誌名</td>
<td>熱帯医学</td>
</tr>
<tr>
<td>年月日</td>
<td>1975-03-20</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10069/4171">http://hdl.handle.net/10069/4171</a></td>
</tr>
</tbody>
</table>

NAOSITE: Nagasaki University's Academic Output SITE
http://naosite.lb.nagasaki-u.ac.jp
Sensitivity of Rapid Plaque Assay Method of Dengue Viruses

Yoshihiro MAKINO and Kumato MIFUNE

Department of Virology, Institute for Tropical Medicine, Nagasaki University

(Chief: Prof. Kaoru HAYASHI)

Abstract: The sensitivity of the hemadsorption-negative plaque test for the infectivity assay of dengue virus type 2 was compared with those of other methods such as conventional plaque test, intracranial inoculation technique of suckling or weanling mice and intrathoracical inoculation technique of Aedes aegypti. The results indicated that the infectivity of the virus can be rapidly and reliably assayed by this method and the sensitivity of the method is higher than that of suckling or weanling mice intracranial inoculation technique, although about 5-fold less sensitive than that of intrathoracical inoculation technique of Aedes aegypti.

Physicochemical, biochemical and growth characteristics of mouse brain passaged dengue viruses have been extensively studied. However, many problems still remain unclear, especially on the pathogenicity of the virus and the pathogenesis of dengue hemorrhagic fever. One of the main reasons of the slow progress in these fields might be due to the lack of adequate virus growth system and rapid assay method of the infectivity of the virus in tissue culture. Various assay methods of the virus in tissue culture system have been reported (1, 2, 3, 4, 6, 12, 14, 16) previously, but they, in general, take long incubation periods to obtain the results and are less susceptible than 2 to 3-day-old suckling mouse intracranial inoculation method.

Recently, rebella virus was demonstrated to be rapidly assayed as hemadsorption-negative plaques using viral interference phenomenon with Newcastle disease virus (7, 10). The mechanism of the interference has been further characterized (5, 8, 9) and applied for the infectivity assay of various arboviruses (9). The present study describes the experiments on the infectivity assay method of dengue virus type 2 and on the sensitivity of the method.

MATERIALS AND METHODS

Viruses: Dengue virus type 1 (Hawaii, mouse brain passage 13), type 2 (Tr-
1751, mouse brain passage 64), type 3 (Ph H–87, mouse brain passage 29) and untyped low mouse brain passaged dengue virus (Saigon–13, mouse brain passage 5) were kindly supplied by Dr. A. Oya, National Institute for Health, Japan. These viruses were used as a form of 10% suspension in phosphate buffered saline (PBS) containing 0.75% bovine serum albumin and antibiotics (pH 7.4). These virus stocks were sealed in small tubes and stored at -75°C and were not thawed until use. Type 2 (D–2) and Saigon–13 were passaged several times in a stable line of porcine kidney (PS) cells and used occasionally in some experiments.

Cell cultures: Stable line of grivet monkey kidney (BSC–1) cells were grown and passaged in Eagle’s minimum essential medium (MEM) supplemented with 10% bovine serum and 1.05g of sodium bicarbonate/liter. Cell monolayers in petri dish were grown in Eagle’s MEM supplemented with 10% bovine serum, 1.4g/liter of sodium bicarbonate and antibiotics and incubated in a 5% CO₂ atmosphere. For maintenance, the medium was replaced to Eagle’s MEM supplemented with 5% calf serum, 2.1g/liter of sodium bicarbonate and 20 units/ml of mycostatin.

PS cells used in the present study were cloned in our laboratory and the most susceptible clone to dengue virus replication was selected. Growth medium consists of Eagle’s MEM supplemented with 10% calf serum, 2.1g/liter of sodium bicarbonate. For maintenance, the medium was replaced to Eagle’s MEM containing 2% fetal bovine serum (FBS) and 2.8g/liter of sodium bicarbonate and incubated in a 5% CO₂ atmosphere.

The growth of established lines of African green monkey kidney (Vero) cells, baby hamster kidney (BHK 21) cells, rabbit kidney (RK 13), and HeLa cells was essentially the same as described above.

Virus assay: (1) Hemadsorption-negative (HAD–) plaque test: This was done as described elsewhere (10) in rubella virus assay. Briefly, BSC–1 cell or other cell monolayers grown in 60 mm petri dish were inoculated with serially diluted virus stock and allowed to adsorb for 1 hr at 37°C in a 5% CO₂ atmosphere. Then the monolayers were flooded with 4.5 ml of maintenance medium and incubated for 72 hr, after which 0.2 ml of 1:30 dilution of Newcastle disease virus (NDV) (10⁶ PFU/ml) were superinfected. The monolayers were incubated for an additional 18 hr at 37°C in a 5% atmosphere. At the end of incubation, the culture medium was discarded and 2 ml of 0.5% sheep red blood cells in physiological saline was added. Within 20 min at room temperature, negative plaques were counted in the background of a confluent sheet of red blood cells adsorbed to the cells in which NDV was grown.

(2) Plaque assay in PS cells: PS cells grown in 60 mm petri dish were inoculated with virus. After 1 hr adsorption at 37°C in a 5% CO₂ atmosphere, 3 ml of the methyl cellulose overlay medium (MC) or agar overlay medium described below were put on the monolayer and incubated for 72 hr. The second overlay medium was then overlaid and plaques were counted on the following day. MC consists of Eagle’s MEM supplemented with 5% calf serum, 1.5 g/liter of sodium bicarbonate, 20 units/ml of mycostatin and 1% methyl cellulose (4,000 cps, Nakarai Chemical Co., Ltd). For the second overlay, MC added with 4
ml of 1:1,000 neutral red/100ml of overlay medium was used. Agar overlay medium consists of Eagle’s MEM supplemented with 1% of special agar-noble (DIFCO), 10% calf serum, 4.9g/liter of sodium bicarbonate and 20 units/ml of mycostatin. The second overlay medium contained neutral red as described above.

(3) Virus assay in mosquitoes: In order to prevent laboratory infection, colonized adult male Ae. aegypti (Bangkok strain, 126th passage level) was used in these experiments. Each mosquito was inoculated intrathoracically with 0.3 to 0.5 µl of 10-fold diluted virus stock with microsyringe under chloroform anesthesia and incubated for 7 days at 28°C. At the end of incubation period, the mosquitoes were harvested and ground in glass homogenizer with 1.5 ml/mosquito of PBS containing 0.75% bovine serum albumin and antibiotics (pH 7.4). After centrifugation of the suspension at 10,000 rpm for 15 min at 4°C, the supernatant was assayed for virus content by HAD- plaque test in BSC-1 cells. ID₅₀ in mosquito was calculated by the method of Reed and Muench (11).

(4) Virus assay in mice: Mice (ICR strain) were inoculated intracranially with 0.02 ml of serially diluted virus stock and observed for sickness or death for 3~4 weeks. LD₅₀ was obtained by the method of Reed and Muench (11).

RESULTS

(1) The relation between virus dilution and the number of HAD- plaques: Fig. 1 shows a linear relationship between the virus (D-2) dilution and the number of plaques in BSC-1 cells. The number of plaques reached maximum by day 3 and did not increase significantly thereafter, although the plaque size became larger. This suggests that one plaque is originally made from single infectious virus particle. HAD- plaque tests were also performed with low or high mouse brain passaged viruses and tissue culture adapted viruses of other type of dengue virus including Saigon-13. All of these viruses formed countable HAD- plaques.

Another established cell lines such as PS cells, BHK 21 cells, HeLa cells and Vero cells were examined for their ability to form HAD- plaques by inoculating appropriately diluted D-2 virus stock under the same condition. However, among these cell lines, only Vero cells were found to form clear and countable plaques after 4 days of incubation, although the sensitivity was about one tenth of that of BSC-1 cells.
(2) The growth of D-2 virus in adult male Ae. aegypti: To examine the virus growth in mosquitoes, preliminary experiment was performed. Each mosquito was inoculated intrathoracically with 0.3 to 0.5 µl of virus solution, which contained about 65 PFU in average, and incubated at 28°C. Every day, 4 mosquitoes were harvested and ground in glass homogenizer with 2 ml of PBS supplemented with 0.75% bovine serum albumin and antibiotics (pH 7.4). After centrifugation of the homogenate at 10,000 rpm for 15 min at 4°C, the supernatant was assayed for infectivity by HAD plaque test. As shown in Fig. 2, the virus multiplied in mosquitoes after one day's latent period, and reached plateau after incubation for 7 days. The titer on 8th day was $2.3 \times 10^3$ PFU/mosquito. Therefore, in the following experiments, the infected mosquitoes were harvested on 8th day of incubation at 28°C.

![Figure 2 Growth of dengue virus type 2 in adult male Aedes aegypti after intrathoracical infection.](image)

(3) ID$_{50}$ in mosquito: To obtain ID$_{50}$ in mosquito, 10-fold serially diluted D-2 virus suspensions were inoculated into groups of mosquitoes as described in Materials and Methods. After incubation for 7 days at 28°C, each mosquito was harvested and examined for virus content. As illustrated in Table 1, the percent of virus recovery from mosquitoes was well correlated with inoculated PFU/mosquito. From the percent of virus recovery, ID$_{50}$ in mosquito was calculated to be $10^{-4.65}$ dilution of the virus which corresponded to 0.19 PFU.

(4) Plaque assay in PS cells: In order to compare the sensitivity to D-2 virus of PS cells with that of BSC-1 cells, both cell monolayers were inoculated at the same time under the same condition. PS cells developed countable plaques after 4 days incubation. The number of plaques formed was almost equal to that of HAD plaques in BSC-1 cells when MC was overlaid. However when agar medium

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>PFU/mosquito</th>
<th>Virus recovery*</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$</td>
<td>8.4</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0.84</td>
<td>8/10</td>
<td>80</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0.08</td>
<td>3/10</td>
<td>30</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>0.008</td>
<td>1/10</td>
<td>10</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>0.0008</td>
<td>0/10</td>
<td>0</td>
</tr>
</tbody>
</table>

* Numerator shows number of mosquitoes virus recovered and denominator number of mosquitoes inoculated.
was overlaid, the number of plaques markedly decreased as illustrated in Table 2.

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>No. of plaque per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agar overlay</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>120</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>15</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0</td>
</tr>
</tbody>
</table>

* The number of plaques is too numerous to count.

It was also noted that almost all of the plaques in PS cells (under either MC or agar medium) were so called “bull's-eye” plaques, central intact cells and surrounding dead cells consisted one plaque.

(5) $LD_{50}$ in mice and the comparison of the sensitivity of $D-2$ virus assay in various hosts: For comparison of the sensitivity of various methods of $D-2$ virus assay, 2-day-old, 9-day-old suckling mice and 2-3 week-old weanling mice were inoculated intracranially with serially diluted stock virus and the titers of $LD_{50}$ obtained were converted into PFU. The results were summarized in Table 3. Sensitivity was expressed as 100-fold of the reciprocal of PFU of $ID_{50}$ or $LD_{50}$. Expectedly, $Ae. aegypti$, which is a main vector of dengue virus, was found to be the most sensitive host and about five times more sensitive than HAD- plaque method in BSC-1 cells. Whereas HAD- plaque test in BSC-1 cells was more sensitive than the method with suckling mice or weanling mice. PS cells showed the same sensitivity as BSC-1 cells when overlaid with MC.

**DISCUSSION**

Many assay methods of the infectivity of dengue viruses have been studied previously using primary tissue culture cells or established cell lines. They can be classified into following 2 groups: The method to observe the cytopathic effects of challenge virus by interferon-mediated interference mechanism (1, 3) and the method by conventional plaque technique.
(2, 4, 12, 16). However, these methods usually require 7 days to 13 days to obtain the results. Recently, a new type of assay method by staining synthesized viral antigens using indirect fluorescent antibody technique was reported (6) and it was demonstrated that the infectivity was assayed after 2 days incubation. This kind of improvement of the assay method of the virus infectivity is considered to be essential for further studies of dengue viruses.

As described previously (9), BSC–1 cells infected with dengue virus developed a refractory state to the superinfection with NDV and formed HAD– plaques after incubation for only 3 days. The number of plaques obtained paralleled the dilution of virus despite of using liquid overlay medium. The sensitivity of this system was higher than that of suckling mice or weanling mice and same as PS cells when MC was overlaid. So far as we studied, adult male Ae. aegypti was the most sensitive host for virus assay. However, this system requires experienced techniques and takes longer time to obtain the results. Preferably, this system should be used with modification (ie: using female instead of male) for detecting a few amount of virus such as the virus in circulating blood.

In PS cells infected with D–2 virus, “bull’s-eye” plaques were observed. This kind of plaque has been reported to be frequently seen in the case of homologous interference between incomplete and complete virus (15). Further study should be done in this field. In PS cells, the plaque formation of D–2 virus was markedly inhibited under agar overlay medium when compared with MC. The inhibitory effect of agar has been investigated in the systems between KB cells and group B arboviruses and it has been shown that in the case of D–2 virus, agar itself inhibits cytopathic effect of the virus and demonstrable multiplication of the virus (13). Further studies are needed in this system to make sure whether or not the inhibitory effect of agar on plaque formation is mediated by a similar mechanism.

When summarized the results, infectivity of dengue viruses can be routinely and rapidly assayed in HAD– plaque test in BSC–1 cells or conventional plaque technique in clonized PS cells under MC with high sensitivity.

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. A. Oya from the National Institute for Health, Tokyo, for his kind supply of dengue viruses and to Prof. Y. Wada, Department of Medical Zoology, Nagasaki University School of Medicine, for his supply of Aedes aegypti. The authors also express their sincere thanks to Prof. K. Hayashi of the Department for his continuous interest and encouragement to this work.

REFERENCES


デンゲウイルスの迅速定量法の感受性について

牧野芳大，三舟求真人 （長崎大学医学研究所ウィルス学部門）

デンゲウイルスの定量法としては，マウス脳内接種法。ブラック法，ウイルスの干渉を利用し，攻撃ウイルスの細胞変性効果の出現の有無をみる方法等があるが，そのいずれも，日数がかかり且つ複雑である。迅速で正確な定量法を確立することは，デンゲウイルスを研究する上で重要である。従来，風疹ウイルスの定量法であるニューヨークコレラウイルスに対する干渉を応用した赤血球吸着陰性（HAD-）ブラック法が，デンゲウイルスをはじめいくつかのアルゴウイルスにも応用できることが既に報告した。今回は，2型デンゲウイルスを用いて，デンゲウイルスをBS C-1細胞を使用し

HAD-ブラック法で定量する方法に検討をおこなえ，さらにクローン化したPS細胞で従来の方法によるブラック法，乳の貯蔵及び乳製品の脳内接種法，デンゲウイルスの生きたベクターであるネッタイシマカの雄成虫への脳内接種法等とその感受性を比較する実験を行ない，以下の結果を得た。（1）HAD-ブラック法を用いると，デングウイルス2型は3日間の培養でHAD-ブラックを形成し，その数と接種ウイルス量とは直線的比例関係を示した。（2）雌ネッタイシマカの脳内接種を行うとき，デンゲウイルスは7目の潜伏期を経て増殖をはじめ，28℃，7日間の培養でウイルス数はほぼ最高に達した。（3）雄ネッタイシマカは80℃を1細胞のHAD-ブラック法と比べて約5倍の感受性を示した。しかしHAD-ブラック法は乳のマウス，離乳マウス脳内接種法より高い感受性があることが判明した。 （4）クローン化したPS細胞は，メチルセルロース培養法を
用いてウイルスをプラック定量すると、BSC−1細胞によるHAD−プラック法とほぼ同じ感受性を示した。しかしPS細胞に寒天重層法を用いて定量するとプラック数は著明に減少した。又、メチルセルロース重層法、寒天重層法のいずれの場合もBull’s eye plaque がみられた。以上の場合から、BSC−1細胞によるHAD−プラック法またはPS細胞によるメチルセルロース重層法は、デングウイルスをかなり高い感受性で、容易に正確に且つ迅速に定量可能な方法であることがわかった。

熱帯医学 第16巻 第4号 163−170頁 1975年2月