BRIFE NOTE

The Effect of Overlay Medium on Plaque Formation of Tissue Culture-Attenuated Newcastle Disease (TCND) Vaccine Virus

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In the plaque technique for enumerating virus infectivity and assaying genetical variants, agar gel overlay and neutral red staining are most commonly employed to localize lesions caused by virus infection [3]. Recently, the plaque characteristics of various strains of Newcastle disease virus (NDV) have been described in detail; the authors reported that the plaque development with lentogenic strains of NDV was poor and seldom obtained by conventional agar overlay methods [5, 6].

Differences in plaque size, number and morphology have been reported to be due to the inhibitory factors in the overlay medium (serum, solidifying agent, etc.) and to the staining procedure [3].

The present study was made to obtain a suitable overlay medium and a staining procedure for simple and reproducible plaque assay of Tissue Culture-attenuated Newcastle Disease (TCND) vaccine virus.

The TCND vaccine virus grown in primary porcine kidney monolayers and used in our experiments was reported previously [8]. Monolayer cultures of chicken embryo fibroblast (CEF) used for the plaque assay were prepared in 60-mm petri dishes from 10-day-old embryos obtained from our specific-pathogen-free flock. The cell sheet in a dish contained approximately $5 \times 10^6$ cells after overnight incubation at $37^\circ C$ in a 5% $CO_2$ atmosphere. The growth medium consisted of Eagle's minimum essential medium (MEM, Nisui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal calf serum (Flow Laboratories Inc., Maryland, USA), and 250 units of penicillin G potassium, 250 mg of dihydrostreptomycin sulfate and 5 mg of fungizone per ml.

Monolayers were rinsed twice with Hanks' balanced saline and inoculated with 0.2 ml of each dilutions of the virus in Eagle's MEM. After 1 hr adsorption at $37^\circ C$, these plates were overlaid with 5 ml of medium. The overlay medium contained the following solidifying agents prepared in Eagle's MEM without phenol red: 1% special Noble agar (Difco Laboratories, Michigan, USA), agarose (Nakari Chemicals, Ltd., Kyoto) or 1.5% methocel (4000 cps, Wako Pure Chemical Industries, Ltd., Osaka) washed with alcohol and ether. Fetal calf serum was supplemented to 5% concentration for the agar and agarose medium, and 2.5% for the methocel one. After 4 days incubation at $37^\circ C$, the cultures were fixed with alcohol-acetic acid-formalin fixative,
Table 1. Effect of overlay medium on plaque formation in CEF monolayers by TCND vaccine virus

<table>
<thead>
<tr>
<th>Overlay medium (Percent concentration)</th>
<th>Average plaque number</th>
<th>Average plaque size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staining procedure</td>
<td>Staining procedure</td>
</tr>
<tr>
<td></td>
<td>Neutral* red</td>
<td>Crystal violet</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neutral* red</td>
</tr>
<tr>
<td>Agarose (1.0%)</td>
<td>124</td>
<td>124</td>
</tr>
<tr>
<td>Agar (1.0%)</td>
<td>48</td>
<td>52</td>
</tr>
<tr>
<td>Methocel (1.5%)</td>
<td>Not done</td>
<td>136</td>
</tr>
</tbody>
</table>

Remarks.
* : Neutral red solution was added approximately 4 hr before observing the plaques.

Fig. 1. Plaques of TCND virus in chicken embryo cell monolayers stained with crystal violet

then stained with 3 ml of 1:100 crystal violet (E. Merck, Darmstadt, Germany) after removal of the overlays as described by Goto et al. [4]. The remaining culture was also stained by adding 4 ml of 1:5000 neutral red (E. Merck, Darmstadt, Germany). Five dishes were used for each virus dilution.

The comparative plaque size and number obtained with the TCND strain of NDV under the different overlays and staining procedures are summarized in Table 1. Plaques under agar and methocel were small, while those formed under agarose were strikingly larger as shown in Fig. 1.

Under the agarose and methocel overlays, the number of plaques produced were approximately equal, but a significantly lesser number was noted under the agar overlay medium. Plaques that developed under the agarose and agar overlays were slightly larger with the crystal violet staining procedure than the neutral red staining. The methocel method made counting the plaques easier, although a linear relationship existed between the plaque number and the relative concentration of the virus under each overlay medium.

These data show that agarose overlay facilitates plaque formation by the TCND strain. Under agarose, plaques are larger
than under the regular agar overlay, and easier to read as under methocel. Also, agarose is as convenient to handle as agar and methocel, and form a firm gel. Shingh et al. [7] used agarose in their overlay for plaque titration of the lentogenic F strain of NDV, which was seldom formed under agar without treatment with additives such as DEAE dextran and Mg++. In sum, agarose overlay greatly enhanced plaque formation by Tissue Culture-attenuated Newcastle Disease (TCND) vaccine virus in chicken fibroblast cell monolayers. In contrast, plaques formed under the regular agar overlay were inhibited in number and smaller in size. Those formed under the methocel overlay were not inhibited but their sizes were small as under the agar.

References