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A Simple Micro-culture Method for the Study of Group B Arboviruses*

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Thirty-nine group B arboviruses have been titrated by a simple micro-culture method. The technique uses a stable line of pig kidney cells (PS cells) in which plaques develop when cells are first infected in suspension in the wells of haemagglutination trays and are then incubated for from 3 to 10 days under an overlay containing carboxymethyl-cellulose. This method can be adapted to measure neutralizing antibodies, and the principle underlying the test is applicable to other cells and other viruses.

A list of 39 separately named group B arboviruses, including the agents responsible for several important diseases of man, is given in a recent report of the WHO Scientific Group on Arboviruses and Human Disease (1967). Several different techniques are available for final identification within the serological group but, of these, virus neutralization tests are generally agreed to be the most specific. Cross-neutralization tests with 39 different viruses and 39 different sera would be excessively laborious in mice, but could be performed in tissue culture if a simple technique were available which could be applied to all the viruses within the group. It is believed that the method to be described fulfils these requirements. This paper describes the method and its application to the titration of infectivity of group B arboviruses. A further paper will present the results of cross-neutralization studies.

Most plaque methods for animal virus studies use either the technique of Dulbecco (1952), in which dilutions of virus are applied to a pre-formed monolayer of cells which are then incubated under a gelled medium, or else that of Cooper (1955), in which cells are infected in suspension and are then incubated in a thin film of agar layered upon a base layer of agar. The method described here uses a different principle. Cells are infected in suspension and are then incubated under conditions which allow the formation of a monolayer in which the concurrent growth of virus leads to the development of virus-induced plaques. Generalized infection of the cell sheet is prevented by the use of a semi-solid gel produced by the incorporation of the sodium salt of carboxymethyl-cellulose (CMC) in the medium. Tests are carried out in Perspex haemagglutination trays of the standard WHO pattern, using Leibovitz medium L15 which enables cells to be grown in free exchange with air without the use of a carbon dioxide incubator. The cells are a line of stable pig-kidney cells, first derived by Inoue & Ogura (1962), and used by Westaway (1966) in a study on 12 group B viruses. The method is an extension of that described by Salim (1967) who applied it to the growth of phlebotomus fever viruses.

MATERIALS AND METHODS

Viruses

The list of group B viruses used is that given in Annex 2 of the report of the WHO Scientific Group on Arboviruses and Human Disease (1967). The strains and mouse passage levels are listed in the accompanying table. Stock preparations of virus were prepared from the brains of infected infant mice which were triturated in an all-glass grinder to give a 10% suspension in normal saline containing 10% of newborn-calf serum, the extract being centrifuged at 10 000 rev/min for 1 hour (6500 *g*) and stored in capillary tubes at −70°C in a dry-ice cabinet.

Cells

PS cells were received from Dr E. G. Westaway, Queensland Institute for Medical Research, Brisbane, Australia. Preliminary cultures were grown in Eagle's medium supplemented with 10% tryptose

* From the National Institute for Medical Research, Mill Hill, London, England.

PLAQUE PRODUCTION BY GROUP B ARBOVIRUSES IN PS CELLS

Virus	Strain	Passage	Highest positive dilution	Days	Plaques	Size (mm)
1. Apoi	Original	7	10^{-9}	6	Clear	4
2. Banzi	H 336	11	10^{-8}	3–5	Clear	3
3. Bukalasa bat	BP 111	6	10^{-7}	7	Poor	1–2
4. Bussuquara	An 4073	9	10^{-6}	6	Clear	4
5. Cowbone Ridge	W-10986	7	10^{-8}	8	Faint	1–2
6. Dakar bat	Dakar 249	5	10^{-6}	6	Poor	1
7. Dengue type 1	Hawaii A	16	10^{-6}	8	Clear	2
8. Dengue type 2	Tr 1751	56	10^{-6}	8	Clear	2
9. Dengue type 3	H-87	29	10^{-6}	6–7	Clear	1–2
10. Dengue type 4	H-241	15	10^{-8}	5–6	Clear	2
11. Edge Hill	C-281	4	10^{-7}	6	Clear	2
12. Entebbe bat	IL 30	25	10^{-8}	6	Clear	4
13. Ilheus	Original	32	10^{-8}	6	Clear	3
14. Israel turkey	Original	31	10^{-7}	5	Clear	2
15. Japanese encephalitis	Nakayama	46	10^{-8}	5	Clear	3
16. Kokobera	MRM 32	6	10^{-6}	6	Faint	1–2
17. Kunjin	MRM 16	8	10^{-8}	3–5	Clear	4
18. Kyasanur forest disease	W 377	6	10^{-8}	6	Clear	2
19. Langat	TP 64	6	10^{-8}	6	Clear	2
20. Louping ill	Moredun	?	10^{-8}	6	Clear	2
21. Montana myotis leukoencephalitis	B 310 A 564	13	10^{-5}	6	Faint	1
22. Modoc	M-564	9	10^{-8}	6	Clear	2
23. Murray Valley encephalitis	1/1951	16	10^{-8}	6	Clear	3
24. Negishi	Original	10	10^{-8}	6	Clear	5
25. Ntaya	Original	15	10^{-7}	6	Clear	2
26. Omsk	Bogoluvoka	24	10^{-8}	5	Clear	2
27. Powassan	Byers	8	10^{-9}	6	Clear	4
28. Spondweni	AR 94	9	10^{-8}	6	Clear	3
29. St. Louis encephalitis	McCallum	6	10^{-6}	5	Clear	2
30. Stratford	C 338	7	10^{-5}	6–10	Faint	1–2
31. Tembusu	M 1775	12	10^{-8}	5	Clear	2–3
32. Tick-borne encephalitis	Hypr	47	10^{-7}	6	Clear	2–3
33. Uganda S	Original	30	10^{-8}	3–5	Clear	2
34. US bat salivary gland	Original	9	10^{-8}	7	Clear	3
35. Usutu	SA AR 1776	8	10^{-8}	3–4	Clear	4–6
36. Wesselsbron	Original	146	10^{-7}	5	Clear	4
37. West Nile	Egypt 101	5	10^{-6}	4	Clear	2–3
38. Yellow fever	17 D	22	10^{-6}	6	Clear	2
39. Zika	MR 766	149	10^{-8}	6	Clear	2–3

phosphate broth and 10% newborn-calf serum, but all later experiments were carried out using Leibovitz L15 medium (Leibovitz, 1963). The standard growth medium was L15 medium supplemented with 10% tryptose phosphate broth and with 3% newborn (pre-colostral) calf serum. Penicillin (100 μg/ml) and streptomycin (100 μg/ml) were used as a routine. Amphotericin B (2 μg/ml) was used in some experiments.

Stock cultures were grown in 8-oz (approx. 230 ml) glass bottles with screw-caps. The medium was discarded from a confluent cell sheet, the cells were rinsed with 20 ml of tris-buffered Gey's solution without calcium and magnesium salts, and 20 ml of a mixture of trypsin and trisodium edetate (Versene; EDTA) was applied (0.125% Difco trypsin + 0.1 g/litre trisodium edetate). The bottle was returned to the incubator for 10 minutes, after which the fluid was decanted and the bottle reincubated without further treatment for an additional 10–15 minutes. When the cells were detached from the glass, either 80 ml of growth medium were added, and the resuspended cells were distributed into 4 8-oz bottles, or 10 ml of growth medium were added and the concentration of cells was determined by direct enumeration in a counting chamber without the addition of a stain. Stock cultures could be divided 1 : 4 every 4 or 5 days. The yield from 1 8-oz bottle was around $0.6–1.0 \times 10^7$ cells.

Cloning of PS cells

As originally received, the PS cells were not homogeneous, and cultures prepared from small inocula showed variation in the size of the colonies which developed and in the size of the individual cells within different colonies. Dilute suspensions of cells were distributed into Petri dishes and, after attachment of the cells, isolated single cells were enclosed within glass rings fixed with silicone grease and incubated until colonies had developed. Six cloned populations were established in this way. Of these, 2 were made up of large cells, and 4 of small cells. The small-cell clones formed smoother monolayers and were less inclined to form multilayered sheets; they also survived longer without a change of medium. The sensitivity to group B arboviruses of both large- and small-cell clones appeared to be the same but plaques were somewhat clearer in cultures made up of the small cells. Most studies were therefore carried out with clone D, one of the small-cell clones.

Plates

Standard Perspex WHO-pattern haemagglutination trays containing 80 wells were sterilized by exposure for 30 minutes to ultraviolet light. A specially constructed box containing 2 UV lights ensured that both sides of the plate were sterile.

Distribution of cells and virus

Cells in growth medium were diluted to a concentration of 3×10^5 cells per ml. Virus dilutions were prepared in the same medium. The unit volume in the test was 0.2 ml, measured by an automatic pipette [1] (Fig. 1). Paper drinking-straws served as convenient disposable pipettes; they were cut into 3 pieces about 7 cm long and sterilized in half test-tubes in a hot-air oven at 160°C for 1 hour. The straws were not plugged, but were attached to the automatic pipette by means of a Teflon adaptor which fitted on to the nozzle of the automatic pipette at one end and received the straw inside it at the other end. A cotton-wool plug in the adaptor prevented contamination of the straw from the pipette, which was not sterile. The adaptor was sterilized at 160°C for 1 hour and was changed with every change of virus, or whenever was required. Straws were changed as required.

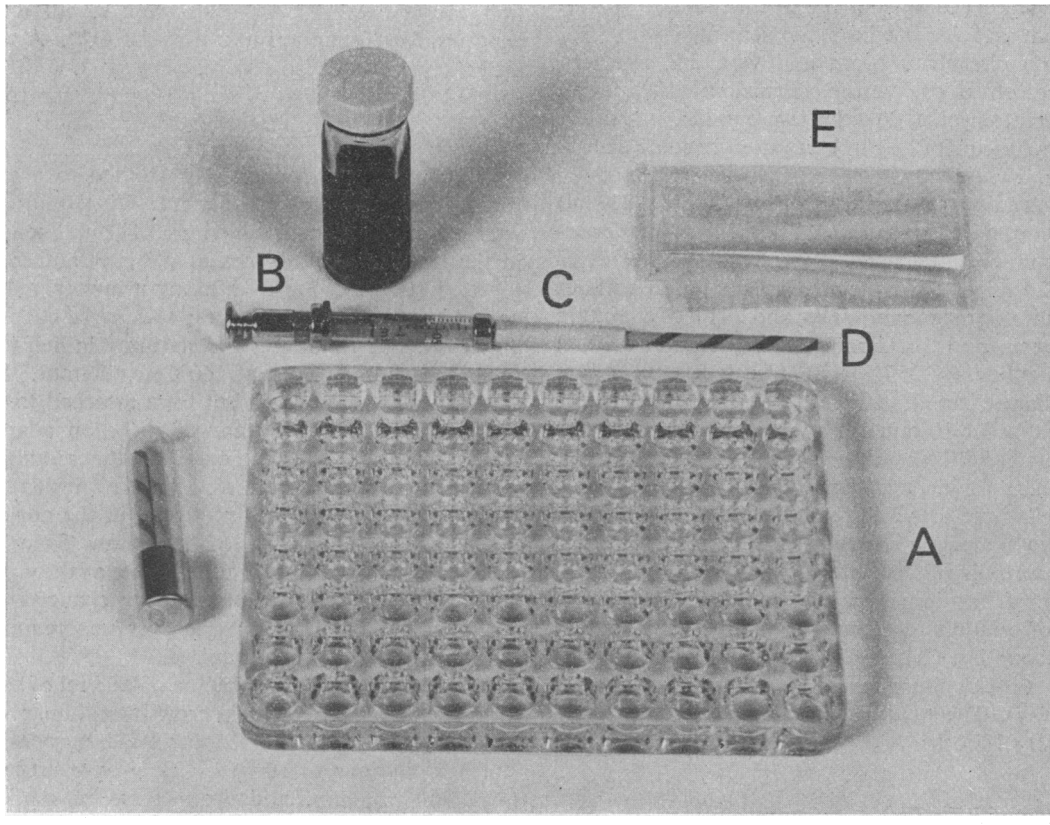
As a routine, an initial volume of 0.05 ml of stock virus was diluted to 5.0 ml in growth medium, giving a dilution of $10^{-3.0}$ of mouse-brain suspension. Further dilutions to $10^{-8.0}$ or $10^{-9.0}$ were prepared in 1.8-ml volumes, and, from these, 6 replicate samples from each dilution were distributed into 1 line of wells in a tray. Control medium without virus was added to 6 wells to the left and right of the rows of virus dilutions. One volume (0.2 ml) of cells (3.0×10^5 cells per ml) was then added to each of the control wells, then to each of the wells containing virus, beginning at the most dilute preparation. The tray was then slid into a polyethylene bag, the open side of which was folded over, and placed in an incubator at 36°C. The polyethylene bags were sterilized by exposure to gamma irradiation.

Addition of overlay

After incubation for 4–5 hours, the tray was removed from the incubator and from its bag, and 0.4 ml of overlay medium was added to each well, using a larger automatic pipette also fitted with a Teflon adaptor and a sterile straw. The tray was placed within another sterile bag and returned to the incubator.

[1] Supplied by R. D. Turner & Co., Ltd.

FIG. 1
APPARATUS USED FOR PLAQUE TITRATION[a]



[a] A = haemagglutination tray; B = automatic pipette; C = Teflon adaptor; D = paper drinking-straw; E = sterile polyethylene bag.

Overlay medium

This was the same as the growth medium, but contained 1.5% of carboxymethyl-cellulose, so that the final concentration of CMC above the cells was 0.75%. It proved impossible to prepare concentrated L15 medium without tryrosine crystallizing out upon storage at 4°C. The following formulation of overlay medium, based on the L15 formula given by Schmidt (1964), was therefore devised:

Carboxymethyl-cellulose (sodium salt, British Drug Houses, Ltd)	1.5 g
L-tyrosine	30.8 mg
Ion-exchange water	50.0 ml

This mixture was autoclaved at 10 lbf per in² (0.7 kgf/cm²) for 10 minutes and to it were then added the remaining constituents of L15 medium, also made up to 50 ml:

Solution A; amino-acids and carbohydrates (10×concentrated)	10 ml
Solution B; L-glutamine, 3 g per 100 ml	1 ml
Solution C; Hanks' BSS (10×concentrated) without carbohydrate or sodium bicarbonate	10 ml
Solution D; Eagle's minimum essential vitamin mixture (100×concentrated)	1 ml
penicillin (10 000 μg/ml) and streptomycin (10 000 μg/ml)	1 ml (each)
ion-exchange water	27 ml

The complete overlay medium was made by adding 10 ml of tryptose–phosphate broth and 3 ml of newborn-calf serum to 100 ml of 1.5% CMC in L15.

Terminal stain

After incubation for 3–10 days, depending upon the virus under study, the preparation was stained to demonstrate plaques. In practice, the tray was placed on its side within a plastic box to allow medium to drain off the cells. The box was filled with normal saline solution and the infected saline was discarded into an antiseptic solution (sodium hypochlorite), leaving the wells free from CMC. The box was then filled with naphthalene-black stain, or else the tray was placed within a shallow trough and the wells were filled with stain which was allowed to act for ½ hour. The stain was then discarded and the tray rinsed in tap-water and dried. The stain was made up as follows (Jackson, 1967):

Naphthalene black (naphthol blue-black, colour index number 20470)	1.0 g
Glacial acetic acid	60 ml
Sodium acetate	13.6 g
Water	to 1000 ml

To reduce the risk of contamination, all culture procedures were carried out inside a safety cabinet. Stained plates could be kept indefinitely, but were normally re-used by filling the wells with undiluted sodium hypochlorite solution, followed by thorough washings in a dilute solution of sodium hexametaphosphate (Calgon), in tap-water and in distilled water.

Neutralization tests

Serial 2-fold dilutions of normal or immune sera were prepared in 0.2-ml volumes in haemagglutination tray wells, using standard growth medium as diluent. A further 0.2 ml of the appropriate dilution of virus, V/1, was added, and the mixtures were incubated overnight at 4°C. The virus dose was determined by preliminary titration as the lowest dilution which produced confluent plaques. The tray also contained serum controls without virus, serial 4-fold dilutions of virus V/1, V/4, V/16, V/64, and V/256, and medium controls. The following morning, 0.2 ml of cell-suspension was added, followed 4–5 hours later by 0.4 ml of overlay medium. Thereafter the procedure was the same as for virus titration.

RESULTS

All the group B arboviruses that have been tested produced plaques in PS cells under the experimental conditions described, although the time of appearance, size, and clarity of the plaques varied with different viruses. The median time for optimal staining of plaques was 6 days, with a scatter down to 3 days and up to 10 days. Banzi, Kunjin, Usutu, Uganda S, West Nile and Wesselsbron viruses gave plaques that were 4 mm–6 mm in diameter on day 6 and were more easily counted if stained on the third or fourth day, at which time plaques were well defined and 2 mm–3 mm in diameter. Dakar Bat, US Bat, Cowbone Ridge and Dengue viruses types 1 and 2 gave small or rather poorly defined plaques on the sixth day, but satisfactory results were obtained by staining on the seventh or eighth day. Several experiments with Bukalasa bat virus have given completely negative results, but poorly defined plaques have developed in some wells infected with 10^{-5} and 10^{-6} dilutions of virus. Further experiments are in progress to define the conditions under which Bukalasa bat virus will produce clear plaques. Stratford virus produced plaques which were less than 1 mm in diameter on day 6, but which increased in size and clarity upon incubation to the ninth or tenth day. The remaining viruses not named fell between the groups represented by Uganda S and Cowbone Ridge, producing medium-sized plaques 1 mm–3 mm in diameter on the fifth or sixth day.

Fig. 2 illustrates a plate of PS cells infected with serial dilutions of Uganda S virus and stained on day 6. Complete destruction of cells is evident at 10^{-3}, 10^{-4} and 10^{-5} dilutions of virus, with some overlapping of plaques at 10^{-6} dilution. Five plaques developed in the 6 wells, each infected with 0.2 ml of a $10^{-7.0}$ dilution of virus, representing a virus titre of 4.2×10^7, or $10^{7.6}$ plaque-forming units per ml of original stock virus preparation.

Fig. 3 illustrates the results obtained when 2 immune sera, S1 and S3, were tested in dilutions from 1 : 10 to 1 : 640 against Langat virus. Duplicate rows were used for each serum and for the controls. S1 did not neutralize the virus at all, whereas there is complete neutralization by S3 at 1 : 10, 1 : 20 and 1 : 40 dilutions of serum, substantial neutralization at 1 : 80 and approximately 50% neutralization at 1 : 160. The wells marked C represent duplicate controls of S1 and S3 sera at 1 : 10 without the addition of virus. Rows 5 and 6 indicate confluent plaques at V/1 and V/4, 5–10 plaques at V/16, 2 plaques at V/64 and 1 plaque in the upper well at V/256. The virus dose in this test was approximately 100 plaque-forming units per 0.2 ml, and the 50% plaque neutralization titre of S1 was <1 : 10, and 1 : 160 for S3.

FIG. 2
PLATE INFECTED WITH SERIAL DILUTIONS OF UGANDA S VIRUS, STAINED ON DAY 6

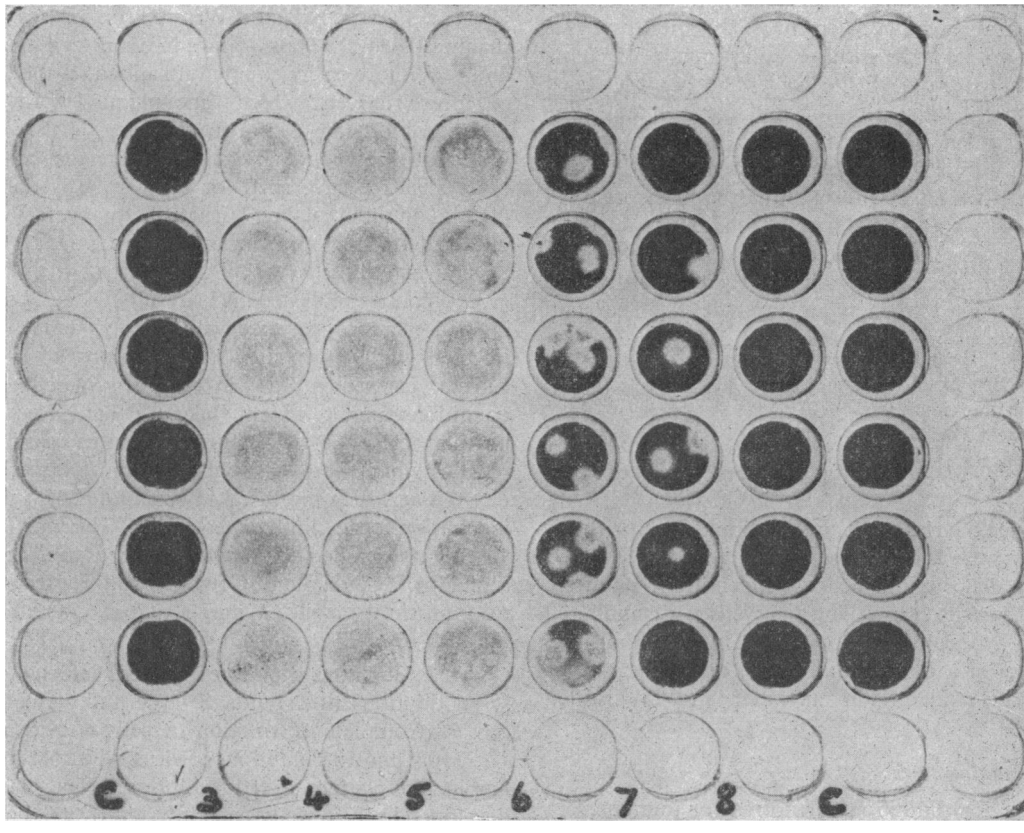
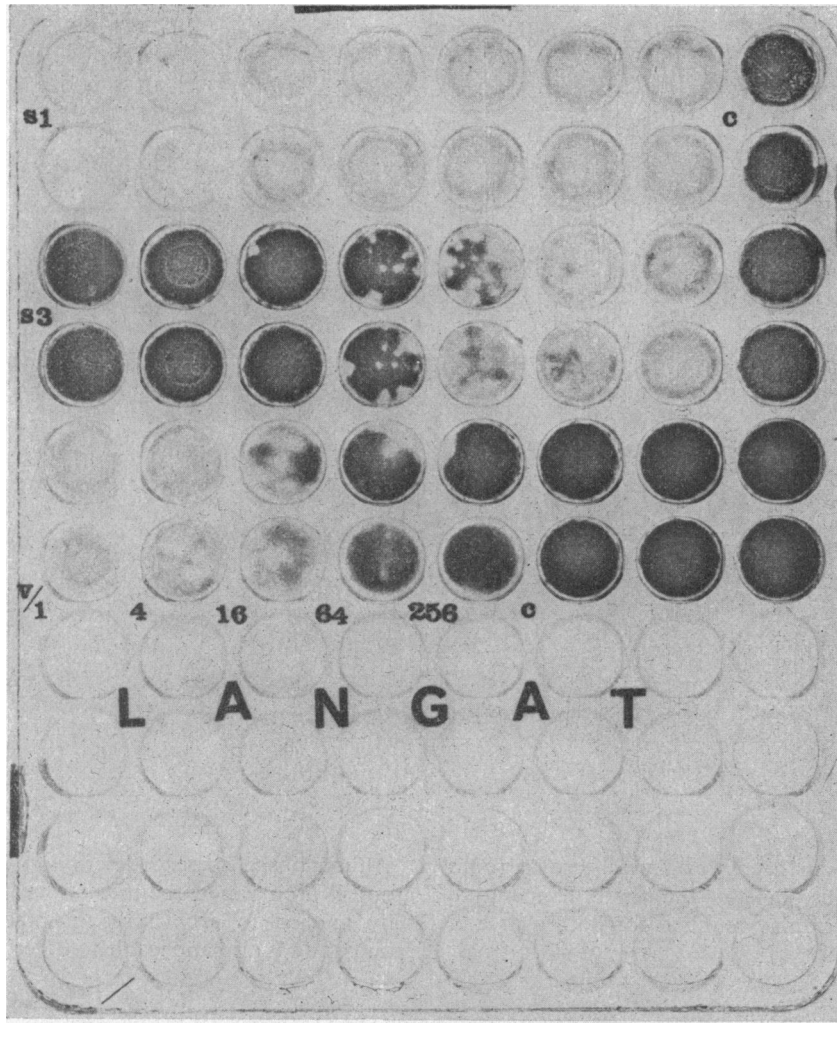


Fig. 4 shows the results obtained with one of the most difficult viruses, Cowbone Ridge, in a plate stained on the eighth day. A prozone effect is apparent, in which high concentrations of virus failed to destroy the cell sheet, although examination under low-power microscopy revealed differences between the control cells and those exposed to high doses of virus. Lower doses of virus produced discrete plaques which were 1–2 mm in diameter. In this experiment, a total of 13 plaques developed in 4 wells each infected with 0.2 ml of a 10^{-7} dilution of Cowbone Ridge virus, representing 1.6×10^8, or $10^{8.2}$ plaque-forming units per ml.

DISCUSSION

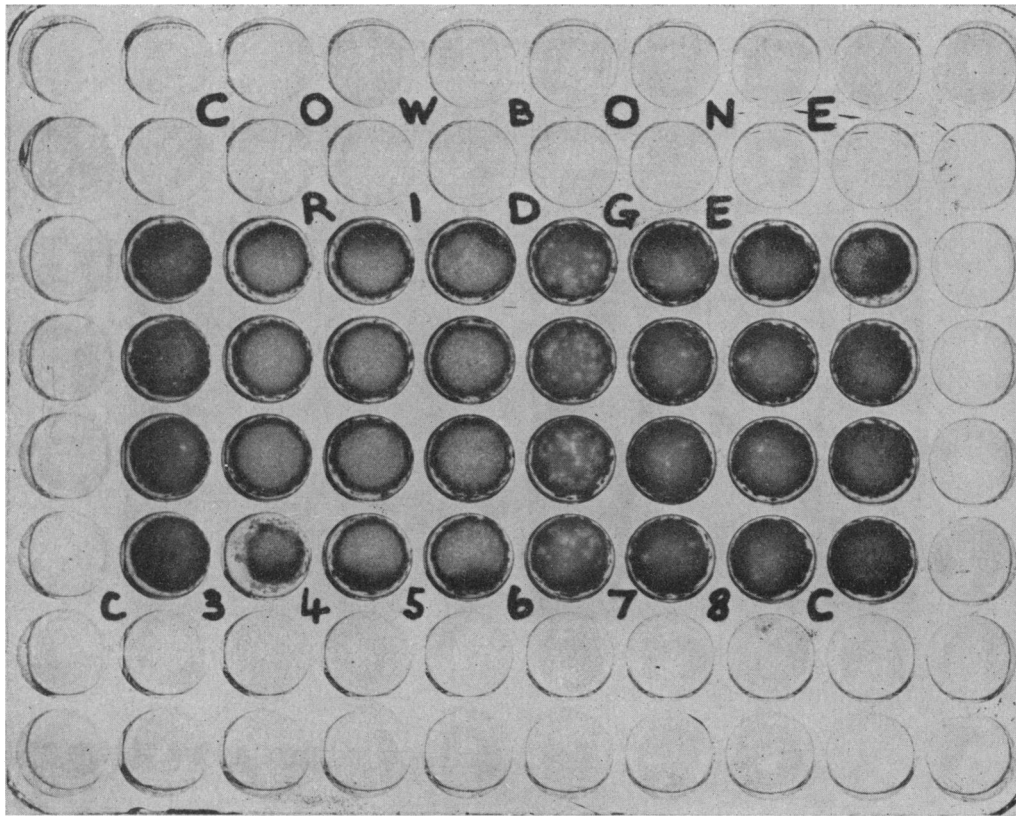
The method described above has proved simple and satisfactory for the study of group B arboviruses, several of which are not readily titrated in other cell culture systems. It is economical on cells and media and uses only simple laboratory equipment. The absence of a requirement for an atmosphere containing added carbon dioxide means that tests can be incubated in a standard laboratory incubator. The principle whereby cells are infected in suspension when they are first seeded into trays eliminates the time normally taken for the formation of a monolayer prior to infection and thus shortens the total time necessary for a plaque assay. The presence of actively dividing cells increases the sensitivity of the assay, and although most group B viruses will form plaques in pre-formed monolayers of PS cells incubated under CMC, plaques are generally larger under the conditions described above. The addition of virus, cells and overlay in succession to the same well without the removal of any fluid simplifies the technique. The use of disposable paper straws as pipettes has proved efficient and economical. The

FIG. 3
NEUTRALIZATION TEST WITH LANGAT VIRUS [a]



[a] First 2 rows: serial dilutions serum S1, 1 : 10–1 : 640; second 2 rows: serial dilutions serum S3, 1 : 10–1 : 640; last well in first 4 rows = C = 1 : 10 of S1 or S3 without virus; fifth and sixth rows: virus controls V/1, V/4, V/16, V/64, V/256, and cell controls.

FIG. 4
PLATE INFECTED WITH SERIAL DILUTIONS OF COWBONE RIDGE VIRUS, STAINED ON DAY 8



CMC overlay can be pipetted with ease at room temperature, thus obviating the careful temperature control of the overlay required for both agar and methyl cellulose.

Although the present description is limited to group B arboviruses, the method has been successfully applied to arboviruses in other serological groups, and is also applicable to other viruses.

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RÉSUMÉ

UNE MÉTHODE SIMPLE DE MICROCULTURE POUR L'ÉTUDE DES ARBOVIRUS DU GROUPE B

On admet généralement que parmi les techniques sérologiques servant à l'identification des arbovirus, les épreuves de neutralisation sont les plus spécifiques. Elles sont cependant d'exécution lente et malaisée lorsqu'on les pratique sur la souris. Un procédé simple utilisant des cultures cellulaires, décrit dans le présent article, a été appliqué à l'étude de 39 arbovirus appartenant au groupe B.

Des cellules rénales de porc obtenues en lignée stable (cellules PS) sont infectées en suspension puis incubées en couche unique sous une couche gélifiée contenant de la carboxyméthylcellulose dans des conditions permettant la formation de plages. Celles-ci sont mises en évidence par coloration après une incubation de 3-10 jours, et l'on peut procéder alors à des épreuves d'inhibition de formation des plages au moyen d'immunsérums. Dans les conditions de l'expérience, tous les arbovirus du groupe B soumis à l'essai ont formé des plages sur cultures de cellules PS, le délai d'apparition des plages, leur taille et leur netteté variant suivant les virus.

Les auteurs soulignent les avantages de la méthode qui ne requiert qu'une faible quantité de cellules et de virus et un appareillage simple. On l'a appliquée avec succès non seulement à l'étude des arbovirus du groupe B mais aussi à l'étude des arbovirus appartenant à d'autres groupes et d'autres virus.

REFERENCES

Cooper, P. D. (1955) *Virology*, **1**, 397-401

Dulbecco, R. (1952) *Proc. nat. Acad. Sci.* (*Wash.*) **38**, 747-752

Inoue, Y. K. & Ogura, R. (1962) *Virology*, **16**, 205-207

Jackson, H. R. (1967) *Canad. J. Microbiol.*, **13**, 117-119

Leibovitz, A. (1963) *Amer. J. Hyg.*, **78**, 173-180

Salim, A. R. (1967) *Trans. roy. Soc. trop. Med. Hyg.*, **61**, 259-264

Schmidt, N. J. (1964) *Tissue culture methods and procedures for viral and rickettsial diseases.* In: Lennette, E. H. & Schmidt, N. J., ed., *Diagnostic procedures for viral and rickettsial diseases*, 3rd ed., New York, American Public Health Association, p. 156

Westaway, E. G. (1966) *Amer. J. Epidem.*, **84**, 439-456

WHO Scientific Group on Arboviruses and Human Disease (1967) *Wld Hlth Org. techn. Rep. Ser.*, **369**